Kinetics of Steady-state Currents and Charge Movements Associated with the Rat Na+/Glucose Cotransporter*

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Mariana Panayotova-Heiermann‡, Donald D. F. Loo, and Ernest M. Wright

From the Department of Physiology, UCLA School of Medicine, Los Angeles, California 90095-1751

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‡To whom correspondence should be addressed: Dept. of Physiology, UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90095-1751. Tel.: 310-825-6905; Fax: 310-206-5661; E-mail: mariana@physiology.medsch.ucla.edu.

Cotransporters are membrane proteins that use the electrochemical potential gradient for ions to accumulate sugars, amino acids, and osmolytes into cells. Using the electrochemical potential gradient for Na+, the Na+/glucose cotransporter (SGLT) accumulates glucose across the brush border membrane of the epithelial cells of the intestine and the proximal tubule of the kidney.

Several members of the SGLT family have been cloned, and these include the high affinity glucose cotransporters (SGLT, \(K_{\text{m}} = 0.2 \text{mM}\) from rabbit small intestine (1) and kidney (2), pig (3), and rat kidney (4), human intestine (5), as well as the low affinity glucose cotransporter (pSGLT2, \(K_{\text{m}} = 2 \text{mM}\) from pig kidney (6)). Mapping the genomic arrangement of the human SGLT1 gene, Turk et al. (7) showed that SGLT1 is a single-copy gene, so that the amino acid sequences from various tissues of a given species are identical. Comparison of the amino acid sequences from the rat, human and rabbit clones revealed 86–87% identity and 93–94% similarity. How does this high degree of homology between the three clones affect their kinetic properties? In this study, we characterized the pre-steady-state and steady-state kinetics of the rat SGLT1 done with a view to understand the relationship between structure and function of members of the SGLT family.

MATERIALS AND METHODS

The pBluescript II SK plasmid containing the coding sequence for rat SGLT1 (4) was linearized with Sall and transcribed in vitro with T3 RNA polymerase (11). The cRNA was overexpressed in Xenopus oocytes and protein function studied 5–10 days after injection using the two-microelectrode voltage clamp (8, 11). To obtain a current-voltage (I-V) relationship, the membrane voltage was stepped for 100 ms to various test values (Vt) between 50 and −150 mV in 20-mV decrements and returned to the holding potential (−50 mV). Averaged currents from three sweeps were low-pass filtered at 500 Hz by an 8-pole Bessel filter and digitized at 100 μs/poin At experiments to study the substrate and cation specificity (see Figs. 3 and 4) the currents were continuously monitored on a chart recorder.

Nonlinear regression analyses were performed using the software ENZFITTER (Elsevier-Biosoft, Cambridge, UK), and the fitting routines in Sigmaplot (Jandel Scientific, San Rafael, CA). The Marquardt-Levenberg algorithm was used by both programs.

Data presented in Figs. 3, 4, and 5A were carried out on the same oocyte. Similar results were obtained by repeating the experiments 2–4 times on oocytes from different donors.

RESULTS

Steady-state Kinetics—Fig. 1A (left panel) shows the current records from a rat SGLT1 cRNA-injected oocyte bathed in the 100 mM NaCl buffer in absence of sugar. The membrane potential was held at −50 mV and then stepped for 100 ms to test potentials (Vt) of 30, −10, −50, and −150 mV. The current relaxation during both the ON and OFF current responses consisted of the capacitive transient followed by a slower decay to the steady state. The slow decay is the pre-steady-state current of SGLT1 and has been observed in the human and rabbit intestinal Na+/glucose cotransporters (8–11). Addition of aMDG (400 μM) to the bath solution generated an inward Na+/current, and abolished the pre-steady-state current (Fig. 1A, right panel). The sugar-induced steady-state current and the pre-steady-state currents were not observed in noninjected oocytes.

The steady-state current-voltage (I-V) relationship of the sugar-induced current is the difference in steady-state current in the absence, and in the presence of aMDG. Fig. 1B shows a family of sigmoidal I-V curves. As seen in aMDG, increased from 31 μM to 20 mM. At each test potential (Vt), increasing [aMDG], increased the sugar-induced current until saturation was reached at 5 mM. For each aMDG concentration, as the test potential was made more negative, the current increased for Vt, between 0 and −100 mV, and then became independent of...
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**Fig. 1. Steady-states kinetics of the αMDG induced transport.** A, membrane current records obtained before and after the addition of 400 μM αMDG to a cRNA rat SGLT1 injected oocyte. The oocyte was clamped at −50 mV. Presented traces are the response to voltage steps (30, −10, −50, −90, and −150 mV) applied for 100 ms. The traces were averaged from three sweeps. B, current-voltage (I-V) relationships of the steady-state currents induced by αMDG. The external Na⁺ concentration ([Na⁺]), was fixed at 100 me while the [αMDG], was varied (in mM: 0.015, 0.031, 0.62, 0.125, 0.250, 0.5, 1, 5, and 20). The I-V relationships were obtained as the difference of the steady-state currents in the presence and absence of αMDG. The □ symbols represent the calculated maximal current (I_max), C, voltage dependence of the apparent affinity for αMDG (K_{αMDG}). At each membrane potential (V_m) the αMDG-induced inward currents (I) were fitted to the equation: I = I_{max}(αMDG)K_{αMDG} - I_{max}[αMDG]) where I_{max} is the apparent maximal current at saturating αMDG concentrations and K_{αMDG} is the sugar concentration at 50% I_{max}. K_{αMDG} was 0.2 mM at −150 mV, increased to 0.46 mM at −30 mV and 1 mM at −10 mV. I_{max} was −265 nA at −150 mV. Similar results were obtained in two other experiments.

The voltage dependence of the apparent K_{0.5} for sugar (K_{0.5}) is shown in Fig. 1C. Between −150 and −50 mV, K_{0.5} was relatively insensitive to membrane voltage. However, between −30 and −10 mV, K_{0.5} increased steeply with depolarizing potentials, from 0.46 ± 0.03 at −30 mV to 1.0 ± 0.2 mM at −10 mV. The calculated maximal current I_{max} at −150 mV for this oocyte was −265 nA.

To determine the Na⁺-dependence of the sugar-evoked currents, the steady-state inward currents were measured as [Na⁺], was varied from 0 to 100 mM while [αMDG], was maintained at 5 mM. Fig. 2A shows the Na⁺-dependent sugar-evoked current at V_m = −30, −50, and −70 mV. At each V_m, the current was described by the Hill equation (see legend to Fig. 2B). There was a steep voltage dependence of the apparent affinity for sodium (K_{Na}). K_{Na} increased from 4 ± 0.6 mM at −150 mV to 40 ± 2 mV at −30 mV. The Hill coefficient (1.8 ± 0.3) was independent of voltage for V_m between −150 to −50 mV, and the I_{max} was −318 nA at −150 mV.

Substrate and Cation Specificity—Fig. 3A shows the currents induced by various substrates with the oocyte membrane potential held at −50 mV. Since the experiment was performed on the same oocyte, the magnitude of the current induced by the different substrates indicates the relative affinity of the cotransporter for the substrates. The currents induced by α-glucose, αMDG, and d-galactose were the largest (~200 nA). The l-isomer of glucose is a poor substrate (5 nA). 3-O-Methyl-d-glucopyranose acts as a substrate with moderate activity (~160 nA). The Na⁺/myo-inositol cotransporter shares high amino acid sequence homology (46% identity) to SGLT1 (12), and myo-inositol has been shown to be a substrate of rabbit SGLT1 (13). Fig. 3A shows that myo-inositol is not transported by the rat SGLT1. The Na⁺/glucose cotransporter also shows high amino acid sequence homology to the Na⁺/nucleoside cotransporter (14). Competition experiments using radioactive tracers showed that uridine inhibited the uptake of αMDG,
Fig. 3. Substrate specificity of the rat SGLT1. A, sugar specificity. Continuous current record from a single rat SGLT1 cRNA-injected oocyte showing the sugar-induced inward currents as the membrane potential was held at -50 mV. The dashed line represents the base-line in 100 mM NaCl. At the time, indicated by the arrows 20 mM of each of the potential sugar substrates were added. The sugars were: D-glucose, L-glucose, 3-O-methyl-D-glucopyranose, α-methyl-D-glucopyranoside, D-galactose, myo-inositol, and β-naphthyl-β-D-glucopyranoside. Prior to the addition of the sugar the oocyte was equilibrated in 100 mM NaCl. After the sugar exposure it was rinsed in 100 mM choline chloride, b-glucopyranose, and LiCl, KCl, and CsCl and the currents induced by addition of 25 mM D-glucose were measured. In the presence of 100 mM KCl, RbCl or CsCl, no detectable inward currents were generated by 25 mM αMDG, indicating that they cannot support sugar transport by rat SGLT1. LiCl was found to be able to support sugar transport (Fig. 4). There was an inward current upon substitution of LiCl for choline. Similar to the Na+ leak, this current was also blocked by 50 μM phlorizin (not shown), and indicates that there is a leak of Li+ by rat SGLT1 in the absence of sugar. This Li+ leak was ~50% of the Na+ leak. The current carried by Li+ in 25 mM αMDG was about 25% (~25 versus ~130 nA, Vh = -50 mV) of the current carried by Na+ suggesting lower affinity for sugar in LiCl, as detected for rabbit SGLT1 (16). At Vh = -150 mV, the αMDG-induced currents were -150 nA in 100 mM Li+ and ~250 nA in 100 mM Na+.

Phlorizin Sensitivity—Phlorizin is a high affinity competitive inhibitor of Na+-dependent glucose transport in renal and intestinal epithelia (Kp50 ~ 10 μM, 17). Fig. 5A shows that addition of 5 μM phlorizin into the bath medium inhibited the currents induced by a saturating concentration of αMDG (5 mM) by 85%. The inhibition was complete at 10 μM phlorizin (data not shown). Fig. 5B shows the voltage dependence of the Kp0.5 for the inhibition by phlorizin (Kp50) studied at external αMDG concentrations of 5 mM and 0.25 mM. Kp50 was 0.9 μM at 5 mM sugar and decreased to 0.09 μM when the αMDG concentration was 250 μM.

To determine the inhibitor constant Kp for phlorizin inhibition (Kp50), we performed a series of Dixon plots. We plotted the reciprocal of the currents (1/I) against the phlorizin concentration. The lines in Fig. 5C were obtained by linear regression on phlorizin inhibition of the steady-state currents (Vh = -150 mV) generated by 1 mM and 0.4 mM αMDG. The lines intersect at a phlorizin concentration of ~0.012 μM. Thus the inhibitory constant Kp50 is 0.012 μM (at -150 mV). It remained slightly voltage dependent in the range ~150 mV to ~50 mV, increasing to 0.053 ± 0.003 μM and 0.030 ± 0.010 μM at -70 mV and -50 mV. The errors are S.E. from three oocytes.
**Fig. 5. Inhibition of the sugar induced currents by phlorizin.** A, continuous current record illustrating the inhibition of the αMDG-induced currents by two different phlorizin concentrations. The dashed line represents the baseline in 100 mM NaCl, the arrows show the time when the substrate or inhibitor were added. V_h = -50 mV. Steady-state currents were measured in 100 mM NaCl containing 5 mM αMDG. Addition of 5 μM phlorizin inhibited the maximal sugar-induced current 84% (Δ - 170 to - 27 nA), whereas 50 μM phlorizin completely blocked the sugar-induced current. B, voltage independence of K_0.5. Shown are the calculated K_0.5 values in the presence of two concentrations of αMDG. For the inhibition at 5 mM αMDG the following concentration of inhibitor were added (in μM): 0.05, 0.1, 0.5, 1, 5, 10, and 20, and for the inhibition at 250 μM (in μM): 0.025, 0.05, 0.1, 0.5, 1, and 5. The current differences between the measured steady-state currents in 5 mM αMDG and those measured in each concentration of the inhibitor in 5 mM αMDG at each V_h were fitted to the equation described in Fig. 1C. Same calculations were repeated for the currents measured at 250 μM αMDG. Note that on reducing the sugar concentration from 5 mM to 250 μM, K_0.5 decreased from -0.9 to -0.09 μM. For both curves error bars are mean of three oocytes. C, Dixon plots of the phlorizin inhibition of αMDG induced currents as function of different phlorizin concentrations. The obtained reciprocal currents (1/I) at each V_h for 0.4 mM or 5 mM αMDG at the following phlorizin concentrations (in μM: 0.01, 0.02, 0.05, 0.1, 0.25, and 0.5) were plotted against these phlorizin concentrations [P2]. The straight lines fit the equation: 1/I = K_0.5[P2]I_max[αMDG] + I_{max}(1 + K_0.5[αMDG]), where K_0.5 is the apparent binding constant for substrate, I_{max} is the maximal current at the applied substrate concentration [αMDG], [P2] is the applied concentration of the inhibitor. Lines obtained for different fixed concentrations of substrate have a different positive slope and cross at the point equivalent to the K_0.5 for a competitive inhibitor. As an example we show the plot at V_h = -150 mV where the K_0.5 was 12 μM.

Presteady-state Charge Movements—In absence of sugar the Na⁺/glucose cotransporter exhibits a presteady-state current after step changes in membrane voltage. The presteady-state current records from rat SGLT1 in Fig. 6A were obtained when the membrane voltage was stepped from the holding (-100 mV) to test voltages 50, -10, -50, and -150 mV. The presteady-state currents were completely blocked by 10 μM phlorizin (data not shown) and were not observed in uninjected oocytes.

The dependence of the relaxation time constant of the ON transients (τ) on test voltage V_h is presented in Fig. 6B. τ decreased monotonically from 13.5 ± 2 ms at -50 mV to 2.6 ± 0.1 ms at 50 mV. In the OFF response, τ was independent of the test voltage V_h and was 53 ± 2 ms over the voltage range -50 to 50 mV. Error bars are S.E. from three oocytes. τ of the oocyte capacitive current (t_c) was independent of the membrane potential (0.6 - 0.8 ms).

Fig. 6C shows the dependence of the total charge (Q, integral of the current transients) on membrane voltage. The curve was
The archetypical member of the Na\(^{+}\)-dependent family of transport proteins is SGLT. SGLTs have been cloned from rabbit, rat, human, and pig. This family also includes the transporters for myo-inositol and nucleosides (18). In this study, we characterized the kinetics of the Na\(^{+}\)/glucose co-transporter cloned from rat kidney. Our goal is to understand the structure-function relations of Na\(^{+}\)-dependent glucose transport by comparing and contrasting the kinetics of highly homologous proteins of this gene family.

**Steady-state Parameters**—The estimated apparent affinities for Na\(^{+}\) and glucose (K\(_{0.5}\) and K\(_{0.5}^{\text{MDG}}\)) for the rat SGLT1 show moderate differences compared to those of rabbit and human transporters for these ligands. Fig. 2B shows that in the voltage range more positive than −50 mV there is a steeper voltage dependence for the binding of the Na\(^{+}\)-ions (40 mV at −30 mV) to the rat transporter. This accounts for higher voltage sensitivities of the apparent K\(_{0.5}\) for αMDG in the same voltage range, shown in Fig. 1A.

Lee et al. (4) found that the K\(_{0.5}^{\text{MDG}}\) was 397 μM at −60 mV and our value of 300 μM is in agreement with the value reported. In this study, the steady-state current induced by αMDG was three times higher than the study of Lee et al. (4), and we were able to obtain the voltage dependence of the K\(_{0.5}\) for sugar and sodium. The stoichiometry from the Hill analysis was 2 Na\(^{+}\):1 sugar molecule, and is similar to that of the rabbit and human (9, 10).

Lee et al. (4) also observed a K\(_{0.5}^{\text{MDG}}\) two orders of magnitude less than the value of 10 μM for rabbit SGLT1 (9). The K\(_{0.5}^{\text{Pz}}\) based on inhibition of 50 μM [\(^{14}\)C]glucose uptake was 0.17 μM. Our estimate of the real inhibitory constant K\(_{i}^{\text{Pz}}\) was 0.02−0.03 μM. It was recently observed (19), that the Na\(^{+}\)-dependent glucose transport system in sheep tracheal epithelium also has a high affinity for phlorizin (K\(_{i}^{\text{Pz}}\) = 0.02 μM). The species differences in the affinity to phlorizin observed here are almost certainly due to differences in the amino acid sequence. For the rat SGLT1, the estimated real K\(_{i}^{\text{Pz}}\) can also be regarded as binding dissociation constant and used in future determination of the number of phlorizin molecules binding per cotransporter molecule. The rat, rabbit, and human clones all exhibit a phlorizin sensitive Na\(^{+}\) leak current, which is about 15−20% of the maximal αMDG-induced current.

**Presteady-state Parameters**—In the rat SGLT1, as in the rabbit (11, 20) and human (8) Na\(^{+}\)-dependent glucose transport systems, we observed transient charge movements in sugar free solutions which were completed within 50 ms. These currents were abolished in all three clones by either addition of sugar substrates or the competitive inhibitor phlorizin. The estimated Q-V curves and the resulting parameters (z, Q\(_{\text{max}}\), and V\(_{0.5}\)) give the functional relation between the moved charge and the membrane potential. z is the average number of net elementary charges (q) apparently moved through a distance (δ) across the membrane (field) in each transporter molecule. This means, that either a single elementary charge moves completely through the membrane electric field, or two elementary charges each move 50% of this distance, or any other combination of negative or positive charges move such that Σqδi = 1. The voltage dependence of the charge movement is shown in Fig. 6C, where Q is plotted as a function of the test potential for the rat SGLT1, and is compared for all three cotransporters in Fig. 7A. There is a displacement of about 40 mV to more negative potentials of the Q-V curves for the rat/human transporters in comparison to the rabbit. Table I shows a comparison of the parameters (z, Q\(_{\text{max}}\), and V\(_{0.5}\)) from fitting the Q-V relations to the Boltzmann equation. The apparent valence of the movable charge is the same for all three transporters (z = 1). V\(_{0.5}\), the voltage at 50% Q\(_{\text{max}}\), was similar (∼−40 mV) for the rat and the human clones, whereas the rabbit clone was about 40 mV more positive. Fig. 7B presents the time constants (τ) of the presteady-state current relaxation of the three clones in the membrane voltage range −50 to 50 mV. τ for the ON currents of human or rat SGLT1 transients decrease progressively as the test voltage was made more positive. In contrast, τ for the rabbit SGLT1 transients increased, reached a maximum at 10 mV, and decreases with more depolarizing potentials. Compared to the τ-V curve of the rabbit, the curves of the human/rat transporters are shifted ∼50 mV to more negative potentials.

The maximal charge Q\(_{\text{max}}\) depends on the level of expression of SGLT1 in the membrane since Q\(_{\text{max}}\) = qzC\(_{r}\), where C\(_{r}\) is the total number of transporters. The maximal steady-state inward Na\(^{+}\) current induced by saturating sugar concentrations (I\(_{\text{max}}\)) is proportional to Q\(_{\text{max}}\) (8, 21). I\(_{\text{max}}\) = kqC\(_{r}\), where k is the apparent turnover number of the transporter. k for rat SGLT1 was 30 s\(^{-1}\) and comparable to that of the human and rabbit (Table I).

**Kinetic Model**—The mechanism of Na\(^{+}\)-dependent sugar transport via rat SGLT1 can be explained by a six-state ordered nonrapid equilibrium kinetic model with mirror symmetry sim-
ilar to the proposed models for the rabbit (20) and human (8) transporters. For reviews see Wright et al. (21, 22). The model assumes that (i) the transporter has a valence of 2; (ii) on both membrane surfaces, the transporter can be empty [C]; loaded with Na$^+$ [CNa2]; or fully loaded with Na$^+$ and sugar [SCNa2]; (iii) the transporter binds two Na$^+$-ions before binding sugar; (iv) C, Na$_2$p, and SCNa$_2$ can cross the membrane; membrane voltages affect Na$^+$ binding to the transporter, and translocation of the empty transporter across the membrane. Simulations predict that for rabbit SGLT1 at $\pm 50$ mV 85% of the transporter molecules in the membrane are bound to Na$^+$ and facing out ([CNa2]$^o$), 5% are empty and facing out ([C]$^o$), and 10% are facing in ([C]$^i$).

According to our kinetic model, the presteady-state currents observed after a depolarizing voltage step are due to the charge transfer involved in the dissociation of external Na$^+$ and the reorientation of the unloaded SGLT1 protein in the membrane:

$$\begin{align*}
0 & \rightarrow \text{CNa}_2^o \rightarrow 0.1 \rightarrow 0.1 \rightarrow 0.1 \\
\text{CNa}_2^o & \rightarrow 0.1 \rightarrow 0.1 \rightarrow 0.1 \\
\text{CNa}_2^i & \rightarrow 0.1 \rightarrow 0.1 \rightarrow 0.1 \\
\text{CNa}_2^o & \rightarrow 0.1 \rightarrow 0.1 \rightarrow 0.1
\end{align*}$$

(Eq. 1)

Computer simulations resulted in a set of rate constants which account quantitatively and qualitatively for the observed presteady- and steady-state kinetics. The results suggest that differences in the kinetics between the rabbit and rat/human cotransporters are due to differences in $k_{12}$ and $k_{16}$ (Table I). Such changes in the rate constants must be due to differences in structure between the isoforms. Aligning the primary amino acid sequences shows that there are different residues at 129 out of 665 positions, and, when conservative substitutions were taken into account (K$^R$; S$^T$; D$^E$; Y$^F$; W; and I$^V$), the location of the putative transmembrane domains is indicated by the lower case letters and underlined (…….). The N- and C-terminals in this secondary structure model (7) are placed on the cytoplasmic side of the plasma membrane.

| SGLT1 | $k_{50}$ | $k_{60}$ | $k_{12}$ | $k_{16}$ |
|-------|---------|---------|---------|---------|
| Rabbit | 100     | 35      | $2.0 \times 10^5$ | 400     |
| Human  | 600     | 25      | $0.14 \times 10^5$ | 300     |
| Rat    | 600     | 15      | $0.45 \times 10^5$ | 300     |

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**Fig. 8. Sequence alignment of the human, rat, and rabbit SGLT1 cotransporters.** The full sequence is given for the human SGLT1, for the rat and rabbit cotransporters only the nonconserved residues are included. Identical and residues similar to that in human (D$^E$, R$^K$, S$^T$, I$^V$, L, and Y$^F$) are replaced by dashes (-). The rabbit residues that are significantly different from those in rat and human, i.e., are polar in either rat or human, are shown in bold italics. The location of the putative transmembrane domains is indicated by the lower case letters and underlined (…….). The N- and C-terminals in this secondary structure model (7) are placed on the cytoplasmic side of the plasma membrane.

**Table I**

Presteady-state rate constants from model simulations

|                | $k_{50}$ | $k_{60}$ | $k_{12}$ | $k_{16}$ |
|----------------|---------|---------|---------|---------|
| Rabbit         | 100     | 35      | $2.0 \times 10^5$ | 400     |
| Human          | 600     | 25      | $0.14 \times 10^5$ | 300     |
| Rat            | 600     | 15      | $0.45 \times 10^5$ | 300     |
V = L = M) this reduces to differences at 76 positions. These 76 are evenly distributed between the N- and C-terminal halves of the protein (Fig. 8), and are mostly confined to hydrophobic loops between the putative transmembrane helices. The cytoplasmic hydrophilic N-terminal and the external loops between helices 5/6 and 11/12 contain 43 of the 76 nonconserved residues.

A clue about the residues that may be important in determining kinetic differences comes from consideration of the residues that are identical in pairs of the three transporters. There are 25 residues shared between human and rat, 17 shared between human and rabbit, and 21 between rabbit and rat. Overall, there are 37 residues that are different between species, and none of these are polar residues. There are no significant differences in differences in kinetics between species, k12 and k16, by determining the three-dimensional protein structure through electrostatic interactions. This could be tested by examining functional properties of clones after either mutating the polar residues or swapping hydrophilic loops between species.

Our conclusion is that the kinetic differences between the human, rat, and rabbit transporters primarily are due to two partial reactions involving binding/dissociation of Na+ ions and translocation of the empty carrier. These differences are probably due to polar residues clustered between helices 5/6 and 11/12.

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