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Sodium Leak Pathway and Substrate Binding Order in the Na^+-Glucose Cotransporter

Xing-Zhen Chen,* Michael J. Coady,# Fairouze Jalal,# Bernadette Wallendorff,* and Jean-Yves Lapointe*
Groupe de recherche en transport membranaire, *Département de Physique and #Département de Physiologie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

ABSTRACT The Na^+-glucose cotransporter (SGLT1) expressed in *Xenopus laevis* oocytes was shown to generate a phlorizin-sensitive sodium leak in the absence of sugars. Using the current model for SGLT1, where the sodium leak was presumed to occur after two sodium ions are bound to the free carrier before glucose binding, a characteristic concentration constant (K_c) was introduced to describe the relative importance of the sodium leak versus Na^+-glucose cotransport currents. K_c represents the glucose concentration at which the Na^+-glucose cotransport current is equal to the sodium leak. As both the sodium leak and the Na^+-glucose cotransport current are predicted to occur after the binding of two sodium ions, the model predicted that K_c should be sodium-independent. However, by using a two-microelectrode voltage-clamp technique, the observed K_c was shown to depend strongly on the external sodium concentration ($[Na^+]_o$): it was four times higher at 5 mM $[Na^+]_o$ than at 20 mM $[Na^+]_o$. In addition, the magnitude of the sodium leak varied as a function of $[Na^+]_o$ in a Michaelian fashion, and the sodium affinity constant for the sodium leak was 2–4 times lower than that for cotransport in the presence of low external glucose concentrations (50 or 100 μM), whereas the current model predicted a sigmoidal sodium dependence of the sodium leak and identical sodium affinities for the sodium leak and the Na^+-glucose cotransport. These observations indicate that the sodium leak occurs after one sodium ion is associated with the carrier and agree with predictions from a model with the binding order sodium-glucose-sodium. This conclusion was also supported by experiments performed where protons replaced Na^+ as a "driving cation."

INTRODUCTION

Cotransporters (or symporters) constitute a class of membrane proteins that use the electrochemical energy of one substrate to accumulate another substrate in a cell. In eukaryote cells, sodium-coupled transporters are widely distributed and the Na^+-glucose cotransporter, found in brush border membranes of renal and intestinal cells (Riklis and Quastel, 1958; Crane et al., 1961), may be considered as the prototype for this class of membrane transporters (Schultz and Curran, 1970; Crane, 1977; Aronson, 1978; Toggenburger et al., 1982; Turner and Moran, 1982a, b; Semenza et al., 1984; Restrepo and Kimmich, 1985, 1986; Moran et al., 1988; Kimmich, 1990; Chenu and Berteloot, 1993; Bennett and Kimmich, 1992, 1996).

cDNAs of renal or intestinal Na^+-glucose cotransporters (SGLT1) have been isolated from rabbit, human, pig, sheep, and rat (Hediger et al., 1987, 1989; Ohta et al., 1990; Tarpey et al., 1994; Lee et al., 1994). SGLT1 expression in *Xenopus laevis* oocytes has allowed extensive steady-state and presteady-state studies with excellent control of membrane potential, external solutions and, in some cases, internal solutions (Umbach et al., 1990; Parent et al., 1992a, b; Loo et al., 1993; Veyhl et al., 1993; Chen et al., 1995, 1996). In addition to most of the properties observed for the intestinal Na^+-glucose cotransporter and for the high affinity Na^+-glucose cotransporter of renal proximal tubules, SGLT1 expression produces a phlorizin- (Pz-) sensitive sodium current in the absence of external sugar (Umbach et al., 1990; Parent et al., 1992a; Chen et al., 1995). This current, which represents 2–10% of the maximal Na^+-glucose current, has been called the sodium leak. Parent et al. (1992b) proposed a six-state model where two sodium ions bind simultaneously to the carrier before glucose or Pz binding (with binding order sodium-sodium-glucose, or N^2G). In this model, the sodium leak, described by two rate constants of relatively small amplitude, was assumed to occur after two sodium ions are bound to the transporter. The sodium leak was not further analyzed until recently when our laboratory (Chen et al., 1995) showed, using the cut-open oocyte technique, that this current demonstrated a strong inward rectification in the presence of symmetrical sodium concentrations. Based on assumptions included in the model (Parent et al., 1992b), a sodium-independent characteristic concentration constant (K_c) was introduced (Chen et al., 1995), which allowed a quantitative comparison between the amplitude of the sodium leak and that of the Na^+-glucose cotransport current. K_c represents the sugar concentration at which these two currents are equal.

However, it is not known whether the sodium leak occurs after one or two sodium ions are associated with the carrier. Also, contrary to the stoichiometric coupling for SGLT1 that has been rigorously established as $2Na^+$-1glucose (Chen et al., 1995), the order of substrate binding remains a matter of controversy despite the numerous studies performed on Na^+-glucose cotransport since the 1960s (see,

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Address reprint requests to Dr. Jean-Yves Lapointe, GRTM, Département de Physique, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada. Tel.: 514-343-7046; Fax: 514-343-7146; E-mail: lapoinj@ere.umontreal.ca.
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for example, Kessler and Semenza, 1983; Restrepo and Kimmich, 1985, 1986; Moran et al., 1988; Kimmich, 1990; Parent et al., 1992b; Wright, 1993, Chenu and Berteloot, 1993). Some of the most useful data regarding substrate binding order came from studies on Pz binding and debinding. Experiments with isolated renal microvillus membranes (Aronson, 1978) showed that Pz binding was sodium-dependent. It was later recognized that two different transport systems seem to be present in renal brush border membrane vesicles: a low affinity system in the superficial cortex (mostly S1 segment) and a high affinity system in juxtamedullary tubules (mostly S3 segment) (Turner and Moran, 1982a, b). The sodium dependence of Pz binding was later observed on intestinal epithelial cells and LLC-PK1 cells (Toggenburger et al., 1982; Restrepo and Kimmich, 1986; Kimmich and Randles, 1988). This suggests that at least one sodium ion binds to the carrier before Pz (or, presumably, glucose) can interact with the carrier. The observations that Pz debinding was also sodium-dependent (Aronson, 1978; Restrepo and Kimmich, 1986; Kimmich and Randles, 1988) are in agreement with the binding order sodium-glucose-sodium (NGN), which has been utilized by Kimmich's group to interpret their experimental data (Restrepo and Kimmich, 1985; Kimmich and Randles, 1988; Kimmich, 1990; Bennett and Kimmich, 1992, 1996). However, the NGN model seemed to be disfavored by the observation that the initial rate of Pz binding was sigmoidal as a function of external sodium concentration ($[Na^+]_o$) (Moran et al., 1988) (see Conclusions for more details). While all the intestinal Na^+-glucose cotransport is dependent on functional SGLT1 (Wright, 1993), several Na^+-glucose cotransporters with different stoichiometries and affinities have been cloned from the kidney (Coady et al., 1990; Wells et al., 1992; Mackenzie et al., 1994). On the other hand, Wright's group has used the model N^2G to fit their experimental data obtained with the cloned rabbit, human, and rat SGLT1 expressed in *Xenopus* oocytes (Parent et al., 1992b; Loo et al., 1993; Hirayama et al., 1994; Panayotova-Heiermann et al., 1995). Despite the limitations associated with the lumping of the two sodium ions binding in a single reaction, it appeared that most of their experimental data can be adequately fitted using this model and choosing appropriate rate constants. In addition, a partially ordered model that comprises these two binding orders (Moran et al., 1988; Chenu and Berteloot, 1993) and a random model (Restrepo and Kimmich, 1986; Moran et al., 1988) were also considered.

The presence of the sodium leak certainly complicates the kinetic interpretation of the transport system, but can provide unique information on sodium binding(s) before glucose binding, and thus could be used to deduce a preferred substrate binding order. In the present study, the sodium leak is characterized by use of a two-microelectrode voltage-clamp technique in *Xenopus* oocytes. It is shown that the NNG model for SGLT1 with the sodium leak occurring after two sodium ions are bound to the carrier is clearly inconsistent with 1) the strong sodium dependency of K_c, and 2) significant differences between the apparent sodium affinities for the sodium leak and for cotransport at low glucose concentrations. It is also found that the sodium leak occurs after one sodium ion is associated with the SGLT1 cotransporter. Among the three possible ordered models, only the model NGN can account for all the observed sodium-leak properties.

MATERIALS AND METHODS

Oocyte preparation

Stage V and VI oocytes were extracted from female *Xenopus* frogs and prepared as previously described (Chen et al., 1996). One day after defolliculation, oocyte nuclei were injected with 300 pg of recombinant eukaryotic expression vector pMT21-SGLT1, which contains a full-length human SGLT1 cDNA (Chen et al., 1995). The nucleotide sequence of our cDNA clone was determined and was found to differ slightly from that previously published (Hediger et al., 1989) in that three nucleotides were altered (C_{364} was converted to A, C_{2226} was converted to T, and G_{2235} was converted to A) without any change in the predicted amino acid sequence. It should be noted that a unique *PstI* site was introduced in the 3′ untranslated region of the protein due to these changes. Injection solution also contained 300 pg of pMT21-rGFP, a fluorescent marker for cDNA expression (Coady et al., 1996b), were injected into the nucleus of *Xenopus* oocytes one day after oocyte preparation. Fluorescent oocytes were selected for experiments three to seven days after incubation at 18°C in a modified Barth's solution (Quick et al., 1992).

Data acquisition and analysis

A two-microelectrode voltage-clamp technique was used as previously described (Coady et al., 1996a). Voltages were clamped using a commercial amplifier (Oocyte Clamp model #OC-725, Warner Instrument Co., Hamden, CT); currents and membrane potentials were simultaneously recorded by a data acquisition system (RC Electronics, Santa Barbara, CA). In experiments using Na^+ to drive glucose transport via SGLT1, the oocyte was superfused at ~1.5 ml/min, with a series of saline-Barth's solutions, consisting of (in mM): NaCl + *N*-methyl-D-glucamine (NMDG)-Cl, 90; D-glucose, 0 to 0.1; KCl, 3; $MgCl_2$, 0.82; $CaCl_2$, 0.74; and Tris-Mes, pH 8.0. In experiments using H^+ to drive glucose transport via SGLT1, the oocyte was superfused at the same rate with saline-Barth's solutions, consisting of (in mM): pH from 5.5 to 8.0; Tris + Mes, 20; NMDG-Cl, 50; D-mannitol + D-glucose, 50; KCl, 3; $MgCl_2$, 0.82; and $CaCl_2$, 0.74.

After 5 min of membrane potential stabilization following microelectrode impalements, oocytes were discarded if the membrane potential was still less negative than −35 mV. The membrane potential was then clamped to the holding potential of −77 mV. In some experiments, 100-ms voltage pulses to −96, −114, and −132 mV were applied and steady-state currents were obtained by averaging signals in the interval from 75 to 95 ms after the initiation of the voltage pulses.

The sodium leak in the absence of glucose (I^0_{leak}) was evaluated as the difference between currents recorded before and after 0.1 mM Pz application. Current changes due to addition of glucose (I^G) were also measured. Strictly speaking, I^G equals the cotransport current only at low external glucose concentrations ($[G]_o$), i.e., at $[G]_o$ much lower than the affinity constant for glucose (K^G_m). At $[G]_o$ comparable to K^G_m, addition of external glucose markedly decreases the sodium leak and I^G corresponds to the cotransport current minus the change in the sodium leak.

Experimental results were expressed in the form of mean ± SEM (*N*), where *N* indicates the number of oocytes obtained from at least two different donors. A paired or unpaired Student's *t*-test was used to compare two sets of data whenever appropriate. The curve-fitting procedures were performed with equal weighting using commercially available software (Fig.P version 6.0, Elsevier-Biosoft, Cambridge, UK).

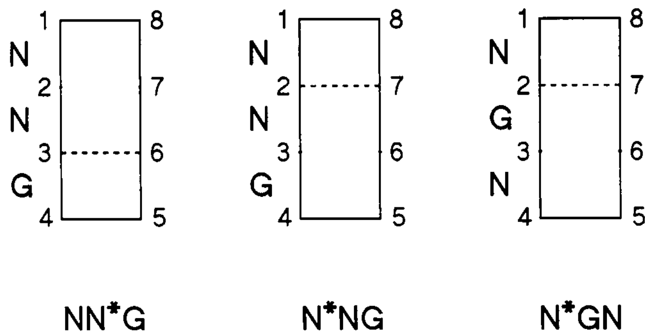

FIGURE 1 Kinetic models with different sodium leak pathways and substrate binding orders. *N*, *G*, and "*" indicate, respectively, sodium, glucose, and the position of the sodium-leak pathway. Sodium leak pathways are shown by dashed lines.

Theoretical results

Based on the facts that 1) the presence of Na^+ is necessary for Pz binding (Pz competes for the glucose binding site) (Toggenburger et al., 1982; Restrepo and Kimmich, 1986; Moran et al., 1988), and 2) a Pz-sensitive sodium leak was observed in the absence of sugars (Umbach et al., 1990; Parent et al., 1992a; Chen et al., 1995), it can be deduced that Na^+ is the first substrate to bind to the transporter. There are only two different orders for the binding of subsequent glucose molecule and sodium ion. However, in the case where two sodium ions are assumed to bind before glucose, two different sodium leak pathways have to be distinguished. This results in the three models (NN*G, N*NG, and N*GN, where "*" indicates the position of the sodium-leak pathway) shown in Fig. 1 [The model N^2G proposed by Parent et al. (1992b) is included in the NN*G model for this purpose].

Characteristics of K_c and the sodium leak

From previous theoretical results (Eq. A40 in Parent et al., 1992b and Eq. A6 in Chen et al., 1995) it can be seen that, for the model NN*G, the sodium affinities for the sodium leak ($K_m^{Na\text{-}leak}$) and for cotransport ($K_m^{Na\text{-}G}$) at low glucose concentrations are equal. In contrast, for both N*NG and N*GN models, $K_m^{Na\text{-}leak}$ and $K_m^{Na\text{-}G}$ at low [G] can be quite different, as a single sodium ion is required for the sodium leak, whereas a second sodium ion is involved in the cotransport process. Under our experimental conditions where the membrane potential is negative, and based on our previous results (Chen et al., 1995), showing that the sodium leak is strongly inward-rectifying and that the affinity for intracellular α-methyl-D-glucose is low (between 25 and 50 mM), the outward components can be neglected. Thus, from the results derived in the Appendix, we summarize the most important characteristics of K_c, I_{leak}^0, and sodium affinity constants in Table 1.

Characteristics of I^G and relation between K_c *and other kinetic parameters*

An expression for the total current similar to Eq. A5 of Chen et al. (1995) can be obtained based on the schematic method of King and Altman (1956). In particular, for the models NN*G and N*GN, the total current (the sodium leak plus the cotransport current) under *zero-trans* conditions can be written in the following form:

$$I = \alpha \cdot ([G]_o + K_c)/(\beta + [G]_o) \tag{1}$$

where α, β, and K_c are independent of $[G]_o$. Then, the current due to glucose addition, experimentally defined as $I^G = I - I_{leak}^0$, can be expressed in the following Michaelis-Menten form:

$$I^G = (I_{max} - I_{leak}^0) \cdot [G]_o/(K_m^G + [G]_o)$$
$$\equiv I_{max}^G \cdot [G]_o/(K_m^G + [G]_o) \tag{2}$$

where $I_{max} = \alpha$, $K_m^G = \beta$, and

$$K_c = K_m^G I_{leak}^0 / I_{max}. \tag{3}$$

The simple approximate relation $K_c = I_{leak}^0/p$, valid at low $[G]_o$ (Eq. A8), where p (= $I^G/[G]_o$) represents the initial slope of the I^G-versus-$[G]_o$ curve, is used in the present study to determine K_c values. Equation 3 is an alternative formula for K_c that is used in cases where the initial slope p is not known.

RESULTS AND DISCUSSION

K_c is sodium-dependent

For each $[Na^+]_o$ (from 2 to 90 mM), solutions containing the following external glucose concentrations (in μM) were used to consecutively perfuse the oocyte chamber: 0, 50, 100, 0, and 0 + 100 μM Pz (Fig. 2 *A*). Total membrane currents at various potentials (−77, −96, −114, and −132 mV) were measured for each solution. Both glucose-dependent currents and the Pz-sensitive sodium leak were obtained by subtraction of currents. As 50 and 100 μM glucose are both much lower than the glucose affinity constant ($K_m^G \approx 1$ mM, Jalal et al., 1996), the I^G-versus-$[G]_o$ curve is assumed to be linear in this range and the initial slope can be determined. By using Eq. A8, the value of K_c was determined by finding $[G]_o$ at which I^G is equal to I_{leak}^0 (Fig. 2 *B*). This simple determination of K_c assumes that Pz acts as a specific inhibitor of SGLT1, which is widely recognized. However, in two recent papers, phlorizin displayed surprising behaviors on Na^+-glucose cotransporters expressed in *Xenopus* oocytes. Lastao et al. (1994) reported on rabbit SGLT1 that Pz may be cotransported with Na^+, based on an observed biphasic effect of Pz on the sodium leak: while inhibition of Pz at 100 μM was more potent than at 5 μM, inhibition with 1 mM Pz was even less potent than with 5 μM. None of the models proposed up to now can

TABLE 1 Comparison of K_c, I_{leak}^0, and sodium affinities for different models

	K_c vs. $[Na^+]_o$	I_{leak}^0 vs. $[Na^+]_o$	$K_m^{Na\text{-}G}$ at low $[G]_o$ and $K_m^{Na\text{-}leak}$
NN*G	independent of $[Na^+]_o$	sigmoidal	equal
N*NG	$= b \cdot ([G]_o + c_1)/[Na^+]_o$,* → 0 at high $[Na^+]_o$	biphasic and → 0 at high $[Na^+]_o$	different
N*GN	$= b \cdot (1 + c_2/[Na^+]_o)$,# → b at high $[Na^+]_o$	Michaelian	different

*$b = k_{27}/2k_{23}^*$ and the concentration constant c_1 is a positive function of rate constants and intracellular glucose concentration ($[G]_i$).
#c_2 is a positive function of rate constants and intracellular sodium concentration ($[Na^+]_i$).

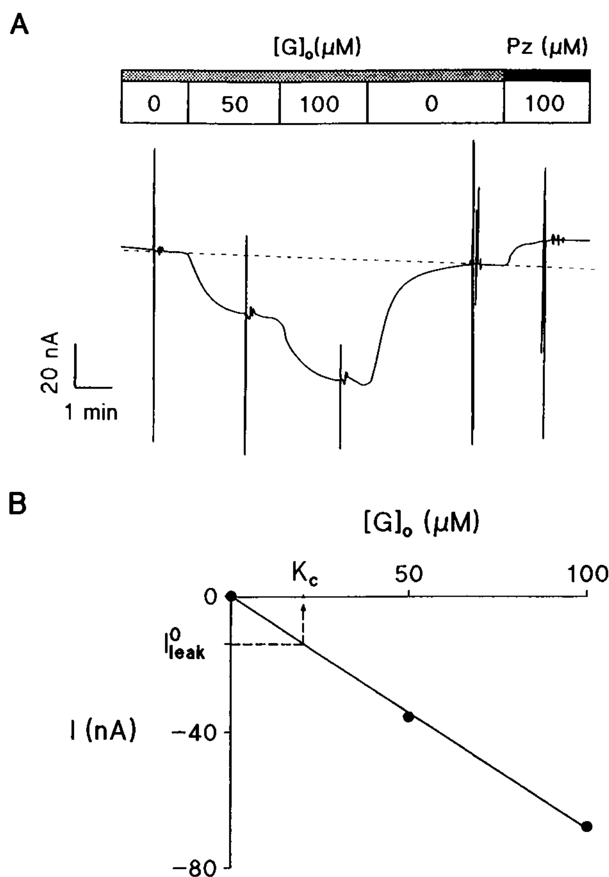

FIGURE 2 Procedure for determining K_c values. (*A*) Example of current recordings in the presence of 50 and 100 μM glucose as well as 100 μM Pz, at a holding potential of -77 mV and with $[Na^+]_o = 20$ mM. Due to the presence of a current drift that may be non-negligible at this current level, the current values were measured relative to the indicated dashed baseline. Following replacement of each solution, a series of voltage pulses (ranging from -77 to -132 mV) were applied. (*B*) K_c value was determined by finding the glucose concentration at which the current due to glucose addition is equal to the sodium leak.

explain this observation: if the maximal Na^+-Pz cotransport current is lower (or higher) than the maximal sodium leak (I^0_{leak}), then measured amplitude of inward current after addition of Pz (a substrate) should be decreased (or increased) monotonously as a function of [Pz]. It can also be seen that, using Eq. 1 where $[Pz]_o$ replaces $[G]_o$, no biphasic effect of Pz is expected on the total current. In agreement with this prediction, we have tested Pz inhibition effect on the sodium leak of the human SGLT1 at five different concentrations, from 10 to 500 μM, and found a monotonous inhibition effect for a variety of $[Na^+]_o$ (2 to 90 mM). More recently, however, Pz was shown to stimulate an inward current instead of inhibiting the inward sodium leak in a chimeric protein formed by an N-terminus of pig SGLT2 and a C-terminus of pig SGLT1 (Panayotova-Heiermann et al., 1996). They concluded that the chimeric protein was able to cotransport Pz with sodium, but they also experimentally confirmed that the native form of pig SGLT1 was unable to transport Pz.

The average K_c for the human SGLT1 at 90 mM external Na^+ was 12.5 ± 1.2 μM ($N = 7$), which is slightly lower than previously published initial estimates (10–60 μM, Chen et al., 1995). In the previous report, K_c was estimated to be 40 ± 12 μM ($N = 3$, two-microelectrode technique) and 28 ± 5 μM ($N = 4$, cut-open oocyte) by an indirect method: the graph of the reversal potential (V_r) versus $[G]_o$ in which K_c was fitted to the theoretical expression in which K_c and stoichiometry ratio (n) are parameters. In the present study, the K_c values increased 10-fold as external sodium concentrations were decreased from 90 to 2 mM (Fig. 3). While the difference between the K_c at 40 mM (15.5 ± 1.3 μM, $N = 9$) and 90 mM was barely significant ($p = 0.045$, $N = 7$), the difference between the K_c at 90 and 20 mM (20.5 ± 1.0 μM, $N = 9$) reached a very significant level ($p < 0.001$, $N = 7$). These results are clearly in contradiction with the prediction from the NN*G model according to which K_c is independent of $[Na^+]_o$ (Eq. A3). These K_c values at various $[Na^+]_o$ were satisfactorily fitted with Eq. A6 derived from the N*GN model and the ratio k_{27}/k^*_{23} and the constant c_2 were found (*solid curve* in Fig. 3), whereas a fit with Eq. A5 from the N*NG model was clearly unsatisfactory (*dashed curve* in Fig. 3). Note that the constant c_2 (20 mM), whose value is close to the sodium affinity constant for cotransport (see Table 2 below at $V_m = -77$ mV), represents the external sodium concentration at which the K_c value had doubled the minimal K_c value at infinite $[Na^+]_o$ (11.6 μM).

We note that, although both the sodium leak and the cotransport current have very complex expressions as functions of rate constants and substrate concentrations, the ratio of these two quantities (I_{leak}/I_{cotr}) can be simply expressed as $K_c/[G]_o$ (Eq. A7) for any one of the three models and at any $[Na^+]_o$. Moreover, at high $[Na^+]_o$, I_{leak}/I_{cotr} is further simplified to $k_{27}/(2k^*_{23}[G]_o)$ for the model N*GN (see Eq.

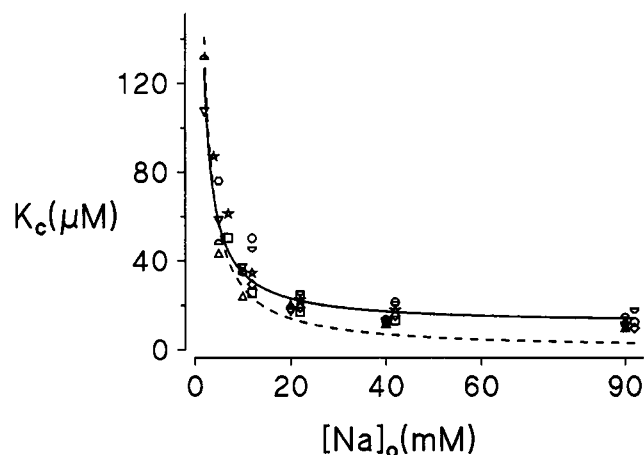

FIGURE 3 K_c values as a function of $[Na^+]_o$ at $V_m = -77$ mV. The curves are fits with the formula $b(1 + c/[Na^+]_o)$, where $b = 11.6 \pm 1.6$ μM and $c = 20 \pm 4$ mM (*solid line*), or with the formula $a/[Na^+]_o$, where $a = 282 \pm 13$ μM^{-1} mM^{-1} (*dashed line*). Data were obtained using nine oocytes from nine different donors.

TABLE 2 Comparison of sodium affinity constants for the sodium leak and for cotransport

V_m (mV)	$K_m^{Na\text{-}leak}$	$K_m^{Na\text{-}G}$ (50)*	$K_m^{Na\text{-}G}$ (100)#
−77	6.8§ ± 0.9 (6)¶	27 ± 2 (5)	30 ± 5 (5)
−96	7.1 ± 0.9 (4)	17.4 ± 0.4 (4)	18.5 ± 0.7 (4)
−114	5.7 ± 1.0 (5)	11.8 ± 0.5 (4)	11.8 ± 0.5 (4)
−132	4.0 ± 0.5 (5)	7.8 ± 0.3 (4)	7.7 ± 0.3 (4)

*Sodium affinity constant for cotransport at 50 μM $[G]_0$.
#Sodium affinity constant for cotransport at 100 μM $[G]_0$.
§in mM.
¶Number of oocytes contributing to average.

A6), i.e., is equal to the leak rate constant (k_{27}) divided by twice the glucose binding rate constant ($2k_{23}^{*}[G]_o$). For the N*NG model, since the translocation corresponding to the sodium leak has to compete with the binding of the second sodium ion, it becomes negligible at high $[Na^+]_o$, and thus $I_{leak}/I_{cotr} \rightarrow 0$ (see Eq. A5).

The sodium leak and Na⁺-glucose cotransport are characterized by different sodium affinities

In the process of determining K_c, I_{leak}^0 and I^G were measured at various $[Na^+]_o$. From these data, the apparent sodium affinities can be obtained both for the sodium leak and for cotransport. At −77 mV, a fourfold difference in sodium affinities can be seen in Fig. 4 where the average I_{leak}^0 (Fig. 4 *A*) and the average I^G in the presence of 50 μM external glucose (Fig. 4 *B*) are shown. Using data obtained with four to six oocytes from different donors at different potentials, the average $K_m^{Na\text{-}G}$ were two to four times larger than $K_m^{Na\text{-}leak}$ (Table 2). Table 2 also shows that the voltage dependence of $K_m^{Na\text{-}G}$ is stronger than that of $K_m^{Na\text{-}leak}$. These differences in affinity and in V_m-dependency are not explicable by the model NN*G (see Table 1). Furthermore, the leak-versus-$[Na^+]_o$ demonstrated a Michaelis-Menten relationship (Fig. 4 *A*), indicating incompatibility with both N*NG and NN*G models. The former predicts a decrease of the leak current at high $[Na^+]_o$, the latter predicts a sigmoidal relation (with the Hill coefficient $n_H > 1$) between the sodium leak and $[Na^+]_o$ (see Table 1). In contrast, all our experimental data were in agreement with predictions of the N*GN model.

H⁺-glucose cotransport supports the N*GN model

Because protons can drive H⁺-glucose cotransport via SGLT1 (Hirayama et al., 1994), we were concerned that H⁺ may contribute to I_{cotr} or I_{leak} at low $[Na^+]_o$, which could significantly affect experimental determination of sodium affinities. Consequently, Pz-sensitive H⁺ currents were measured in several oocytes at different external pH values in the absence of Na⁺. It was found that, at pH 7.5, the Pz-sensitive proton current was practically negligible, which supported our contention that the Pz-sensitive currents previously measured at pH 8.0 were purely sodium currents. With protons replacing sodium, we consider whether the properties of K_c for proton (K_c^H) and of the Pz-sensitive proton leak would be similar to those observed with Na⁺. The Pz-sensitive proton leak and glucose-dependent proton currents for $[G]_o$ = 1–50 mM were measured at pH 6.5 and pH 5.5, allowing K_m^G and I_{max}^G to be evaluated according to Eq. 2 (Fig. 5 *A*). Because in some cases the initial slope of the I^G-versus-$[G]_o$ curve was not available, instead of using Eq. A8 (or the procedure described in Fig. 2 *B*), we determined K_c^H values by using Eq. 3. H⁺ leak turned out to be relatively more important than the sodium leak in acidic external solutions as a proportion of the corresponding maximal cotransport currents. The average leak represented 17 ± 1% (N = 4) and 7.4 ± 0.4% (N = 4) of maximal H⁺-glucose cotransport currents at pH 6.5 and pH 5.5, respectively, at membrane potentials between −77 and −132 mV, compared to ~2% in the case of the sodium leak. K_c^H value is strongly proton-dependent, as K_c^H at pH 6.5

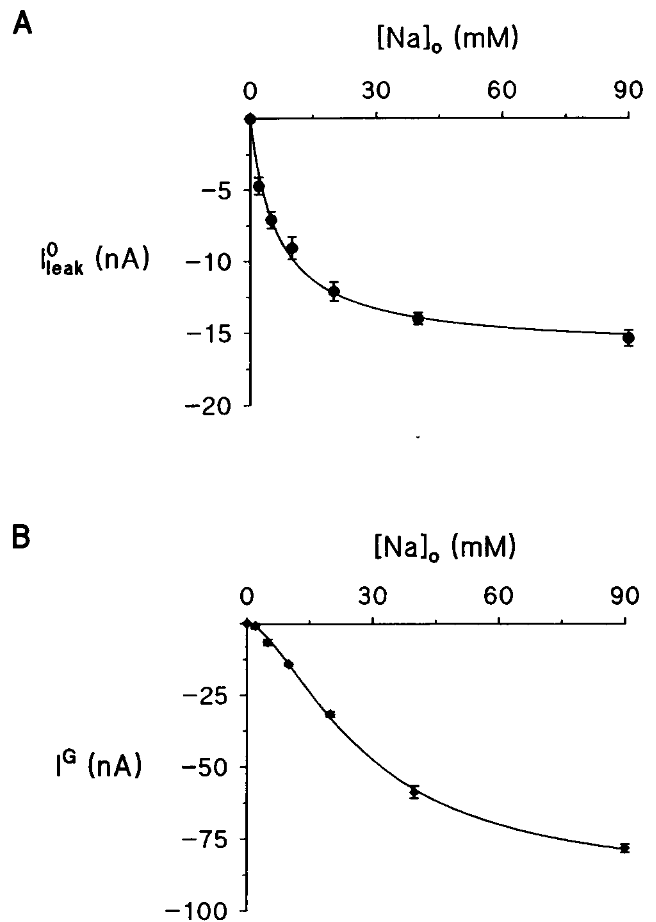

FIGURE 4 Comparison of sodium affinity constants for the sodium leak and for cotransport at V_m = −77 mV. (*A*) Average sodium leak as a function of $[Na^+]_o$, N = 5. The curve represents the best fit of the data to the Michaelis-Menten kinetics with $K_m^{Na\text{-}leak}$ = 6.5 ± 0.8 mM. (*B*) Average sodium currents due to addition of 50 μM glucose for $[Na^+]_o$ ranging from 2 to 90 mM, N = 4. The curve is the best fit to the Hill equation with $K_m^{Na\text{-}G}$ = 28.0 ± 1.6 mM and n_H = 1.64 ± 0.09.

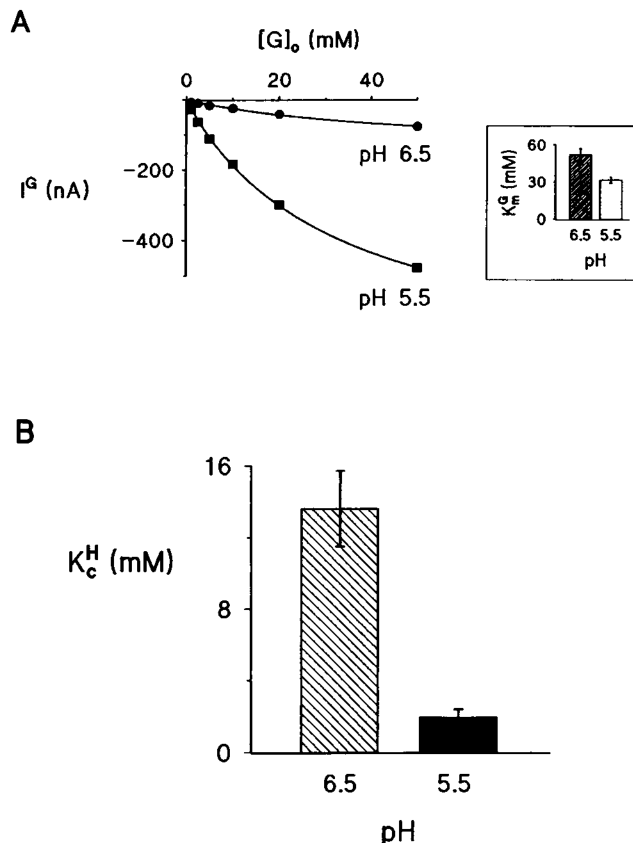

FIGURE 5 Glucose affinity constants for H^+-glucose cotransport and K_c for proton (K_c^H) at membrane potential of −77 mV, pH 6.5 and pH 5.5. (*A*) Proton currents due to glucose addition as functions of $[G]_o$. *Inset:* Averaged glucose affinity constants of 52 ± 5 mM (N = 3) and 32 ± 3 mM (N = 4) were obtained at pH 6.5 and pH 5.5, respectively. (*B*) Individual K_c^H values were determined by using Eq. 3; average K_c^H values were 14 ± 2 mM (N = 4) and 2.0 ± 0.4 mM (N = 4) at pH 6.5 and pH 5.5, respectively.

is sevenfold higher than the one at pH 5.5 (Fig. 5 *B*), which is in accordance with the dependence of K_c on $[Na^+]_o$ reported above (Fig. 3).

When the proton leak was measured in three oocytes, each had a H^+ affinity constant for the H^+ leak ($K_m^{H\text{-leak}}$) of 0.4 μM H^+ (or pH 6.3), which was much lower than that for H^+-glucose cotransport ($K_m^{H\text{-}G}$) in the presence of 1 mM extracellular glucose. From the data presented in Fig. 6, one can state that $K_m^{H\text{-}G}$ (at 1 mM $[G]_o$) > 3.2 μM (or pH < 5.5). This eightfold difference in proton affinities is in accordance with the results using sodium ions (Fig. 4). However, one can see from Fig. 6 that H^+ leak versus $[H^+]_o$ was sigmoidal rather than Michaelian. We believe that protons are likely to play roles other than acting as "driving cations" in interacting with the cotransporter. Recently, it was found that the activity of Na^+-glucose cotransporter at 150 mM Na^+ appeared maximal at pH 7.0 and was remarkably reduced at pH < 6.5 or pH > 7.5 (Oulianova, 1996). Thus, experimental data using protons were in agreement with the results using sodium, supporting our contention that the

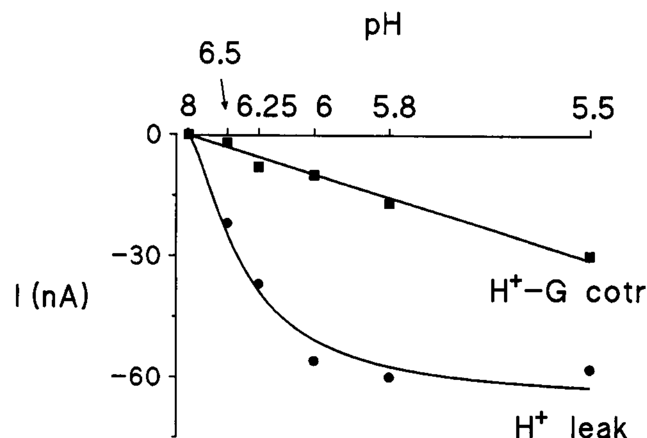

FIGURE 6 Comparison of proton affinity constants for the H^+ leak ($K_m^{H\text{-leak}}$) and for H^+-glucose cotransport ($K_m^{H\text{-}G}$) at V_m = −77 mV. While $K_m^{H\text{-leak}}$ in this example was found to be 0.45 ± 0.07 μM (or pH 6.3 ± 0.1), $K_m^{H\text{-}G}$ at $[G]_o$ = 1 mM must be higher than 3.2 μM (or pH < 5.5) because the measured H^+-glucose cotransport current was linear over the entire pH range used (from pH 8.0 to pH 5.5).

sodium leak (or the H^+ leak) occurs after one Na^+ (or one H^+) is bound to the carrier and that the model N*GN is appropriate for SGLT1.

CONCLUSIONS

Determination of the preferred order of substrate binding in SGLT1 has remained a matter of debate for a number of years. The presence of the sodium leak complicates the kinetics of the transport system but opens a window to the binding process. By comparing the Pz-sensitive sodium leak to the glucose-dependent sodium currents, we determined the sodium-dependence of the characteristic concentration constant K_c that asymptotically decreased, but did not vanish at high $[Na^+]_o$; we also found that a plot of the sodium leak versus $[Na^+]_o$ displayed Michaelian kinetics with the affinity constant considerably lower than that for cotransport at low $[G]_o$. The SGLT1 kinetic model with binding order N*GN, where the sodium leak occurs after one Na^+ is bound, can account for all these observations.

In the experiment on LLC-PK1 by Moran et al. (1988), the initial rate of Pz binding was estimated by measuring the bound Pz at t = 10 s and was found to be sigmoidal as a function of $[Na^+]_o$, contrary to the simple Michaelian relationship predicted by the NGN model. This would represent the only data disfavoring the NGN model up to now. However, this observation could be debated, as it is constantly observed that the Pz inhibition of Na^+-glucose cotransport currents takes effect immediately after Pz application, while measured Pz binding to Na^+-glucose cotransporters has a characteristic time ranging from ~ 10 s to a few minutes (Tannenbaum et al., 1977; Aronson, 1978; Moran et al., 1988; Koepsell et al., 1990; Oulianova, 1996). This suggests that additional steps (possibly sodium-dependent ones) are involved in the process of reaching a state where bound Pz

becomes experimentally detectable, i.e., resistant to washing. We would also like to point out that the apparent sodium affinity constant for Pz binding was found to be >500 mM, which is above all sodium concentrations used in their experiments and nearly 20 times higher than the sodium affinity constant for Na^+-glucose cotransport.

In the present study, we have determined that the N*GN model is the only simple model consistent with our observations. It should be recognized that a more complicated model involving, for example, a random binding of sodium and glucose following the binding of the first sodium would also be possible. However, to be consistent with a Michaelian sodium leak as a function of $[Na]_o$, the binding of the second sodium would have to generate a peculiar sodium leak. For example, whenever the second sodium ion had randomly bound before glucose, a sodium leak involving 2 sodium ions would have to occur with rate constants corresponding to one-half of the rate constants associated with the one sodium ion leak. Future experiments will show whether this more elaborate model is required to describe the functions of SGLT1 expressed in *Xenopus* oocytes.

APPENDIX

Derivation of expressions for K_C

Under steady-state conditions, the occupation probabilities E_4 and E_5 can be expressed as functions of E_2 and E_7 in the case of the NN*G model, or as functions of E_3 and E_6 in the cases of the N*NG and N*GN models (see Fig. 1); thus the sodium leak (I_{leak}) and the cotransport current (I_{cotr}) can be written as follows:
for the NN*G model,

$$\begin{aligned} I_{leak} &= 2N_t e \cdot (k_{63}E_6 - k_{36}E_3) \\ I_{cotr} &= 2N_t e \cdot (k_{54}E_5 - k_{45}E_4) \\ &= 2N_t e \cdot (k_{63}E_6[G]_i - k_{36}E_3[G]_o)/K_c \end{aligned} \quad (A1)$$

and for both N*NG and N*GN models,

$$\begin{aligned} I_{leak} &= N_t e \cdot (k_{72}E_7 - k_{27}E_2) \\ I_{cotr} &= 2N_t e \cdot (k_{54}E_5 - k_{45}E_4) \\ &= N_t e \cdot (k_{72}E_7[G]_i/K_c^i - k_{27}E_2[G]_o/K_c) \end{aligned} \quad (A2)$$

where N_t denotes the number of carriers and $e = 1.6 \cdot 10^{-19}$ Coulomb (elementary charge constant). Factor "2" arises from the fact that two sodium ions are transported across the membrane. For NN*G model, we found (Chen et al., 1995) that

$$K_c = k_{36}(k_{43}k_{56} + k_{54}k_{43} + k_{45}k_{56})/k^*_{65}k_{45}k_{56}, \quad \text{i.e., sodium-independent.} \quad (A3)$$

For the models N*NG and N*GN, the following expressions apply

$$K_c = k_{27} \cdot \det/(2k^*_{23}k^*_{34}k_{45}k_{56}k_{67}\,[Na^+]_o)$$

and (A4)

$$K_c^i = k_{72} \cdot \det/(2k^*_{76}k^*_{65}k_{54}k_{43}k_{32}\,[Na^+]_i)$$

where $\det = k^*_{65}k_{54}k_{43}k_{32}[S]_i + k^*_{34}k_{45}k_{56}k_{67}[S]_o + k_{54}k_{43}k_{32}k_{67} + k_{56}k_{43}k_{32}k_{67} + k_{56}k_{45}k_{32}k_{67}$. For the model N*NG, [S] = [G]; for the N*GN model, $[S] = [Na^+]$. k_{ij} is the rate constant for the transition from state i to state j; k^*_{ij} is defined by $k_{ij} = k^*_{ij}[S]$ with $[S] = [Na^+]$ or [G], when the transition $i \rightarrow j$ represents the binding of "S" to the transporter. The units of k_{ij} and k^*_{ij} are in s^{-1} and $s^{-1}\,M^{-1}$, respectively.

From Eqs. A1 and A2, we see that K_c (K_c^i) corresponds to the $[G]_o$ ($[G]_i$) at which the inward (outward) sodium leak flux is equal to the inward (outward) coupled sodium-glucose flux. When the outward components are neglected (see Theoretical Results, Materials and Methods), K_c corresponds to the $[G]_o$ at which the sodium leak equals the cotransport current ($I_{leak} = I_{cotr}$). We see from Eq. A4 that K_c for both N*NG and N*GN models depends on $[Na^+]_o$:

for the model N*NG,

$$K_c = b([G]_o + c_1)/[Na^+]_o, \quad \text{i.e., inversely proportional to } [Na^+]_o \quad (A5)$$

and for the model N*GN,

$$K_c = b(1 + c_2/[Na^+]_o), \quad \text{i.e., decreases to } b \text{ when } [Na^+]_o \rightarrow \infty \quad (A6)$$

where $b = k_{27}/2k^*_{23}$ and the concentration constant c_1 (or c_2) is a positive function of rate constants and $[G]_i$ (or $[Na^+]_i$). From Eq. A1 or Eq. A2, neglecting outward components, we have

$$I_{leak}/I_{cotr} = K_c/[G]_o. \quad (A7)$$

When $[G]_o$ is much lower than the glucose affinity constant, $I_{leak} \approx I^0_{leak}$, and $I_{cotr} \approx I^G$, then the above relation can be rewritten as

$$K_c = I^0_{leak} \cdot [G]_o/I^G \equiv I^0_{leak}/p \quad (A8)$$

where p represents the initial slope of the I^G-versus-$[G]_o$ curve.

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REFERENCES

Aronson, P. S. 1978. Energy-dependence of phlorizin binding to isolated renal microvillus membranes. *J. Membr. Biol.* 42:81–98.

Bennett, E., and G. A. Kimmich. 1992. Na^+ binding to the Na^+-glucose cotransporter is potential-dependent. *Am. J. Physiol.* 262:C510–C516.

Bennett, E., and G. A. Kimmich. 1996. The molecular mechanism and potential dependence of the Na^+/glucose cotransporter. *Biophys. J.* 70:1676–1688.

Chen, X.-Z., M. J. Coady, F. Jackson, A. Berteloot, and J.-Y. Lapointe. 1995. Thermodynamic determination of the Na^+/glucose coupling ratio for the human SGLT1 cotransporter. *Biophys. J.* 69:2405–2414.

Chen, X.-Z., M. J. Coady, and J.-Y. Lapointe. 1996. Fast voltage-clamp discloses a new component of presteady-state currents from the Na^+-glucose cotransporter. *Biophys J.* 71:2544–2552.

Chenu, C., and A. Berteloot. 1993. Allosteric and Na^+-D-glucose cotransport kinetics in rabbit jejunal vesicles: compatibility with mixed positive and negative cooperativities in a homo-, dimeric, or tetrameric structure and experimental evidence for only one transporter protein involved. *J. Membr. Biol.* 132:95–113.

Coady, M. J., X.-Z. Chen, and J.-Y. Lapointe. 1996a. rBAT is an amino acid exchanger with variable stoichiometry. *J. Membr. Biol.* 149:1–8.

Coady, M. J., G. Lemay, and J.-Y. Lapointe. 1996b. Use of green fluorescent protein cDNA as a marker for nuclear injection of *Xenopus laevis* oocytes. *FASEB J.* 10:A89.

Coady, M. J., A. M. Pajor, and E. M. Wright. 1990. Sequence homologies among intestinal and renal Na^+-glucose cotransporters. *Am. J. Physiol.* 259:C605–C610.

Crane, R. K. 1977. The gradient hypothesis and other models of carrier-mediated active transport. *Rev. Physiol. Biochem. Pharmacol.* 78: 99–159.

Crane, R. K., D. Miller, and I. Bihler. 1961. The restrictions on possible mechanisms of intestinal active transport of sugars. *In* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academic Press, New York. 439–449.

Hediger, M. A., M. J. Coady, T. S. Ikeda, and E. M. Wright. 1987. Expression cloning and cDNA sequencing of the Na^+/glucose co-transporter. *Nature.* 330:379–381.

Hediger, M. A., E. Turk, and E. M. Wright. 1989. Homology of the human intestinal Na^+/glucose and *E. coli* Na^+/proline cotransporters. *Proc. Natl. Acad. Sci. USA.* 86:5748–5752.

Hirayama, B. A., D. D. F. Loo, and E. M. Wright. 1994. Protons drive sugar transport through the Na^+/glucose cotransporter (SGLT1). *J. Biol. Chem.* 269:21407–21410.

Jalal, F., M. J. Coady, M. Cartier, B. Wallendorff, G. Lemay, and J.-Y. Lapointe. 1996. Functional studies of a chimeric Na^+/glucose cotransporter containing portions of SMIT and SGLT1. *FASEB J.* 10:89*a*. (Abstr.).

Kessler, M., and G. Semenza. 1983. The small intestinal Na^+, D-glucose cotransporter: an asymmetric gated channel (or pore) responsive to $\Delta\Psi$. *J. Membr. Biol.* 76:27–56.

Kimmich, G. A. 1990. Membrane potential and the mechanism of intestinal Na^+-dependent sugar transport. *J. Membr. Biol.* 114:1–27.

Kimmich, G. A., and J. Randles. 1988. Na^+-coupled sugar transport: membrane potential-dependent K_m and K_i for Na^+. *Am. J. Physiol.* 255:C486–C494.

King, E. L., and C. Altman. 1956. A schematic method of deriving the rate laws for enzyme-catalyzed reactions. *J. Phys. Chem.* 60:1375–1378.

Koepsell, H., G. Fritzsch, K. Korn, and A. Madrala. 1990. Two substrate sites in the renal Na^+-D-glucose cotransporter studied by model analysis of phlorizin binding and D-glucose transport measurements. *J. Membr. Biol.* 114:113–132.

Lastao, M. P., B. A. Hirayama, D. D. F. Loo, and E. M. Wright. 1994. Phenylglucosides and the Na^+-glucose cotransporter (SGLT1): analysis of interactions. *J. Membr. Biol.* 142:161–170.

Lee, W. S., Y. Kanai, R. G. Wells, and M. A. Hediger. 1994. The high affinity Na^+/glucose cotransporter. *J. Biol. Chem.* 269:12032–12039.

Loo, D. D. F., A. Hazama, S. Supplisson, E. Turk, and E. M. Wright. 1993. Relaxation kinetics of the Na^+/glucose cotransporter. *Proc. Natl. Acad. Sci. USA.* 90:5767–5771.

Mackenzie, B., M. P. Heiermann, D. D. F. Loo, J. E. Lever, and E. M. Wright. 1994. SAAT1 is a low affinity Na^+/glucose cotransporter and not an amino acid transporter. *J. Biol. Chem.* 269:22488–22491.

Moran, A., L. J. Davis, and R. J. Turner. 1988. High affinity phlorizin binding to the LLC-PK_1 cells exhibits a sodium:phlorizin stoichiometry of 2:1. *J. Biol. Chem.* 263:187–192.

Ohta, T., K. T. Isselbacher, and D. B. Rhoads. 1990. Regulation of glucose transporters in LLC-PK1 cells: effects of D-glucose and monosaccharides. *Mol. Cell. Biol.* 10:6491–6499.

Oulianova, N. 1996. Evaluation du modèle dimétrique de cotransport Na^+/D-glucose pour l'interprétation des études cinétiques du transport de glucose et de la liaison de phlorizine sur vésicules de membrane à bordure en brosse isolées du cortex rénal de lapin. *In* Ph.D. Thesis, University of Montreal, Montreal, Canada. 205–225.

Panayotova-Heiermann, M., D. D. F. Loo, C.-T. Kong, J. E. Lever, and E. M. Wright. 1996. Sugar binding to Na^+-glucose cotransporters is determined by the carboxyl-terminal half of the protein. *J. Biol. Chem.* 2701:10029–10034.

Panayotova-Heiermann, M., D. D. F. Loo, and E. M. Wright. 1995. Kinetics of steady-state currents and charge movements associated with the rat Na^+/glucose cotransporter. *J. Biol. Chem.* 270:27099–27105.

Parent, L., S. Supplisson, D. D. F. Loo, and E. M. Wright. 1992a. Electrogenic properties of the cloned Na^+/glucose cotransporter. I. Voltage-clamp studies. *J. Membr. Biol.* 125:49–62.

Parent, L., S. Supplisson, D. D. F. Loo, and E. M. Wright. 1992b. Electrogenic properties of the cloned Na^+/glucose cotransporter. II. A transport model under nonrapid equilibrium conditions. *J. Membr. Biol.* 125:63–79.

Quick, M. W., J. Naeve, N. Davidson, and H. A. Lester. 1992. Incubation with horse serum increases viability and decreases background neurotransmitter uptake in *Xenopus* oocytes. *Biotechniques.* 13:357–361.

Restrepo, D., and G. A. Kimmich. 1985. Kinetic analysis of mechanism of intestinal Na^+-dependent sugar transport. *Am. J. Physiol.* 248: C498–C509.

Restrepo, D., and G. A. Kimmich. 1986. Phlorizin binding to isolated enterocytes: membrane potential and sodium dependence. *J. Membr. Biol.* 89:269–280.

Riklis, E., and J. H. Quastel. 1958. Effects of cations on sugar absorption by isolated surviving guinea pig intestine. *Can. J. Biochem. Physiol.* 36:347–362.

Schultz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes. *Physiol. Rev.* 50:637–718.

Semenza, G., M. Kessler, M. Hosang, J. Weber, and U. Schmidt. 1984. Biochemistry of the Na^+, D-glucose cotransporter of the small intestinal brush-border membrane. *Biochim. Biophys. Acta.* 779:343–379.

Tannenbaum, C., G. Toggenburger, M. Kessler, A. Rothstein, and G. Semenza. 1977. High-affinity phlorizin binding to brush border membranes from small intestine: identity with (a part of) the glucose transport system, dependence on the Na^+-gradient, partial purification. *J. Supramol. Struct.* 6:519–533.

Tarpey, P. S., S. P. Shirazi-Beechey, and R. B. Beechey. 1994. Molecular characterization of the Na^+/glucose cotransporter from the sheep parotid gland acinar cell. *Biochem. Soc. Trans.* 22:264S.

Toggenburger, G., M. Kessler, and G. Semenza. 1982. Phlorizin as a probe of the small-intestinal Na^+, D-glucose co-transporter: a model. *Biochim. Biophys. Acta.* 688:557–571.

Turner, R. J., and A. Moran. 1982a. Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Membr. Biol.* 67:73–80.

Turner, R. J., and A. Moran. 1982b. Further studies of proximal tubular brush border membrane D-glucose transport heterogeneity. *J. Membr. Biol.* 70:37–45.

Umbach, J. A., M. J. Coady, and E. M. Wright. 1990. The intestinal Na^+/glucose cotransporter expressed in *Xenopus* oocytes is electrogenic. *Biophys. J.* 57:1217–1224.

Veyhl, M., J. Spangenberg, B. Büschel, R. Poppe, C. Dekel, G. Fritzsch, W. Haase, and H. Koepsell. 1993. Cloning of a membrane-associated protein which modifies activity and properties of the Na^+-D-glucose cotransporter. *J. Biol. Chem.* 268:25041–25053.

Wells, R. G., A. M. Pajor, Y. Kanai, E. M. Wright, and M. A. Hediger. 1992. Cloning of a human cDNA with similarity to the sodium-glucose cotransporter. *Am. J. Physiol.* 263:F459–F465.

Wright, E. M. 1993. The intestinal Na^+/glucose cotransporter. *Annu. Rev. Physiol.* 55:575–589.