Local osmotic gradients drive the water flux associated with Na^+/glucose cotransport

Pierre-Pascal Duquette, Pierre Bissonnette, and Jean-Yves Lapointe*

Groupe de Recherche en Transport Membranaire, Physics Department, Université de Montréal, Montréal, QC, Canada, H3C 3J7

Edited by Maurice B. Burg, National Institutes of Health, Bethesda, MD, and approved January 29, 2001 (received for review May 26, 2000)

It recently was proposed [Loo, D. D. F., Zeuthen, T., Chandy, G. & Wright, E. M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 13367–13370] that SGLT1, the high affinity intestinal and renal sodium/glucose cotransporter carries water molecules along with the cosubstrates with a strict stoichiometry of two Na^+, one glucose, and ≈220 water molecules per transport cycle. Using electrophysiology together with sensitive volumetric measurements, we investigated the nature of the driving force behind the cotransporter-mediated water flux. The osmotic water permeability of oocytes expressing human SGLT1 (L_p ± SE) averaged 3.8 ± 0.3 × 10^{-4} cm·s^{-1} (n = 15) and addition of 100 μM phlorizin (a specific SGLT1 inhibitor) reduced the permeability to 2.2 ± 0.2 × 10^{-4} cm·s^{-1} (n = 15), confirming the presence of a significant water permeability closely associated with the cotransporter. Addition of 5 mM α-methyl-glucose (αMG) induced an average inward current of 800 ± 10 nA at −50 mV and a water influx reaching 120 ± 20 pL cm^{-2}·s^{-1} within 5–8 min. After rapidly inhibiting the Na^+/glucose cotransport with phlorizin, the water flux remained significantly elevated, clearly indicating the presence of a local osmotic gradient (Δπ) estimated at 16 ± 2 mOsm. In short-term experiments, a rapid depolarization from −100 to 0 mV in the presence of αMG decreased the cotransport current by 94% but failed to produce a comparable reduction in the swelling rate. A mathematical model depicting the intracellular accumulation of transported osmolytes can accurately account for these observations. It is concluded that, in SGLT1-expressing oocytes, αMG-dependent water influx is induced by a local osmotic gradient by using both endogenous and SGLT1-dependent water permeability.

Until the 1990s, it was believed that water movement across cell membrane occurred solely by diffusion through the lipid bilayer and through unidentified pores capable of containing a file of several water molecules (1). In 1990, expression of the facilitated glucose transporter (GLUT) in *Xenopus* oocytes was reported to produce a large increase in membrane water permeability (2), suggesting its capacity of mediating water movement. In 1993, with the cloning of the first water channel (3), it was clearly demonstrated that some membrane proteins do specialize in mediating water transport. Later on, expression of the Na^+/glucose cotransporter (SGLT1) in oocyte, on top of increasing the passive water permeability (4), also was suggested to perform a secondary-active water transport (5). Although the coupling between solute and water transport has long been explained with the generation of a transmembrane osmotic gradient (6), it was proposed that SGLT1 was, in fact, transporting Na^+, glucose, and water in a fixed stoechiometric ratio of two Na^+/one glucose/200–250 water molecules (5, 7–9).

Using oocytes expressing human SGLT1, we present a series of volumetric and electrophysiological experiments aimed at identifying the driving force behind the water movement that accompanies the cotransport of Na^+ and glucose. The main observations are consistent with the presence of transport-related osmotic gradients that can account for all of the observed water flux through the cotransporter.

Materials and Methods

Oocytes and Solutions. *Xenopus laevis* oocytes preparation and cRNA injection were as described (10, 11). Our control solution for volumetric and electrophysiological experiments contained: 50 mM NaCl, 3 mM KCl, 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$, 5 mM Hepes (pH 7.6), and 85 mM mannitol (final osmolality of 197 mosm/kg H_2O). Hypotonic solutions were obtained by removing 50 mM mannitol (final osmolality of 147 mosm/kg H_2O), α-Methylglucose (αMG) solution was obtained by replacing 5 mM mannitol by 5 mM αMG and, when present, 100 μM phlorizin (Pz, Sigma) was directly added to either one of these solutions from a concentrated stock solution in ethanol (20 mM). The osmolality of each solution was controlled within 1 mOsm/kg H_2O with a vapor pressure osmometer (Advanced DigiMatic Osmometer, model 3D2, Advanced Instruments, Norwood, MA). For all experimental protocols, oocytes were equilibrated for at least 20 min in control solution to obtain a stable volume measurement.

For experiments with potassium channel expression, oocytes were injected with 4–6.5 ng cRNA coding for rat ROMK2 (kindly provided by J. Xu and S. C. Hebert, Vanderbilt University, Nashville; see ref. 12) and were incubated at 18°C for 3–4 days in antibiotic supplemented Barth's solution. Experimental solution for these experiments were enriched in K^+ and contained: 50 mM NaCl, 20 mM KCl, 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$, 20 mM mannitol, and 5 mM Hepes (pH 7.6).

Electrophysiology. Oocyte currents were recorded by using the two-electrode voltage clamp technique as described (13, 14). Electrode tips were filled with a cushion layer of 4% agarose in 1 M KCl to reduce the cell swelling associated to KCl leakage into the cytosol (15). Data acquisition and analysis were performed with PCLAMP 6 (Axon instruments, Foster City, CA) and commercially available spreadsheet software.

With SGLT1-expressing oocytes, the membrane potential was clamped at −50 mV, and the current values were sampled at every second (unless otherwise stated). With ROMK2-expressing oocytes, the membrane potential was clamped near the resting potential (≈−35 mV, in the presence of 20 mM external K^+) and an inward current of 1–2 μA was rapidly generated by hyperpolarizing the oocyte by 10 mV. The reversal potential was determined by briefly switching off the voltage clamp.

Optical Measurements. The experimental chamber (volume of 0.07 ml) was similar to the one described by Zeuthen *et al.* (8). The solution flux was 1.2 ml/min, and the dead space between the solution selector and the bath was reduced to 110 μl. Experimentally, 80% of the maximal cotransport current generated by addition of 5 mM αMG could be reached within 20 s. The light

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: αMG, α-methyl-glucose; Pz, phlorizin.

See commentary on page 3628.

*To whom reprint requests should be addressed at: Physics Department, Université de Montréal, P. O. Box 6128, succ. ''centre-ville,'' Montréal, QC, Canada. H3C 3J7. E-mail: jean-yves.lapointe@umontreal.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.071245198

source of 2 mm by 5 mm was touching the surface of the solution to avoid distortion and reflection.

The oocyte volume (V) was calculated from its measured cross-section (A) by using an inverted microscope, a ×3 objective and a video camera (model CCD72, Dage–MTI, Michigan, IN). The system was calibrated with a 1-mm diameter steel ball replacing the oocyte. Images were sent to a computer (Intel 403E 486 30 MHz) through a video card (Image-1280, Matrox Electronic Systems, Dorval, QC, Canada), and our custom-made Windows-compatible software gave a real-time graphic of the number of pixels corresponding to the oocyte cross section. This setup provides a sensitivity of 0.05% in the determination of the oocyte volume. In long time course experiments (10–15 min), a rate of 1 volume measurement per s was generally used, and, in short time course experiments (2–5 min), the accuracy was further improved by taking five measurements per s and averaging the data by bins of 10 points.

Analysis. Assuming a spherical oocyte that swell uniformly, the water fluxes per unit of membrane area (J_w) were calculated as:

$$J_W = \frac{1}{S}\frac{dV}{dt}, \quad [1]$$

where S is a standard oocyte surface of 0.4 cm^2 for an infolding factor of 8.5- to 9.5-fold (16). The water permeability (L_P) in $cm \cdot s^{-1}$ is calculated from the water flux and the osmotic gradient as:

$$L_p = \frac{J_W - J_0}{\Delta\pi}\frac{1}{\bar{v}_W}, \quad [2]$$

where J_W is the water flux per unit of membrane area ($cm^3 \cdot cm^{-2} \cdot s^{-1}$) during the application of an osmotic gradient $\Delta\pi$ ($osmol \cdot cm^{-3}$), J_0 is the baseline water flux observed immediately before the osmotic shock (same units as J_W), and $\bar{V}_w$ is the water-specific molar volume (18 $cm^3 \cdot mol^{-1}$).

All numbers are given as means ± SEM (n), where n is the number of oocytes tested. All experiments were performed by using oocytes from at least two different donors. Paired and unpaired Student's t tests, as appropriate, were used to compare sets of data.

Results

Basic Observations. The effects of SGLT1 on oocyte water permeability were measured by recording the oocyte swelling rate in hypotonic solution for both SGLT1-injected and noninjected oocytes. When calculating the membrane water permeability according to Eq. **2**, noninjected oocytes gave $2.12 \pm 0.14 \times 10^{-4}$ $cm \cdot s^{-1}$ ($L_P^{control}$, $n = 21$) but $3.8 \pm 0.3 \times 10^{-4}$ $cm \cdot s^{-1}$ (L_P^{SGLT1}, $n = 15$) in SGLT1-expressing oocytes ($P < 0.001$). As reported (5), the water permeability of SGLT1-expressing oocytes was reduced to a level not different from noninjected oocytes when 100 μM Pz was added to the bathing solution ($L_P^{SGLT1+Pz} = 2.2 \pm 0.2 \times 10^{-4}$ $cm \cdot s^{-1}$, $n = 15$). Thus, we confirmed that SGLT1 increases the oocyte water permeability (by 1.8- ± 0.3-fold) and that water is very likely to pass through the cotransporter itself as this water flux is totally sensitive to the presence of a specific competitive inhibitor.

Fig. 1 shows a representative example of the long time course swelling induced by the activation of Na^+/glucose cotransport in SGLT1-expressing oocytes. When 5 mM αMG was given in equimolar replacement for mannitol, an average inward Na^+ current (I_{COTR}) of 800 ± 10 nA ($n = 11$) was generated along with a water flux (J_W) reaching 120 ± 20 pL $s^{-1} \cdot cm^{-2}$, 5–8 min after having presented the substrate. Using a stoichiometry of 2 Na^+ ions per αMG molecule (10), this steady-state water flux corresponds to 815 water molecules per αMG transported. An

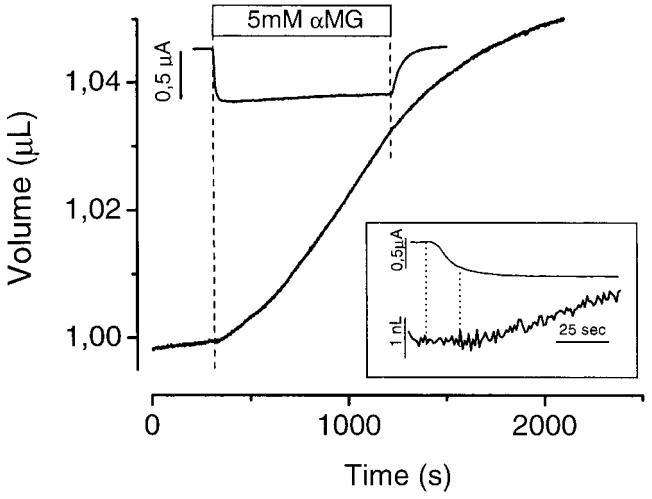

Fig. 1. αMG-dependent water flux. Oocyte current and volume are displayed as a function of time. From t = 300–1,200 s, 5 mM αMG was added to the bathing solution. (*Inset*) Enlargement of the initial portion of the curves. Dotted lines, represent the time needed to reach 80% of the final cotransport current.

enlargement of the initial swelling is shown in the *Inset*. It can be seen that 80% of the maximal cotransport current is reached within 20 s after αMG addition and the volume measurement departs from the initial value at about the same time (taking into consideration the noise level that was estimated to be ± 0.03% in the present case). Upon complete removal of αMG, the cotransport current stops, but the oocytes are still swelling at a high rate (see Fig. 1), which can continue for 15 min and more. The fact that a portion of J_W remained while I_{COTR} vanished shows that the glucose uptake into the oocyte wasn't isotonic and that a significant osmolyte accumulation had occurred during the 15-min exposure to αMG.

Testing the Water Cotransport Hypothesis. *Prolonged period of Na^+/αMG cotransport.* The transport-dependent osmotic gradient was evaluated from the water flux remaining immediately after blocking the cotransporter with 100 μM Pz at the end of a 15-min cotransport period (see Fig. 2). For each oocyte of this series, the membrane water permeability was measured in the presence ($L_P^{SGLT1+Pz}$) and in the absence of Pz (L_P^{SGLT1}). Basically, the cell swelling that continues immediately after Pz addition during glucose cotransport allows for the determination of the effective osmotic gradient at this moment (knowing the value of $L_P^{SGLT1+Pz}$). This osmotic gradient then can be used to predict the passive portion of the water flux in the presence of αMG (using the value of L_P^{SGLT1} measured for this given oocyte). Subtracting this passive water flux from the total water flux in the presence of αMG yields the magnitude of the putative secondary active transport of water. As shown in the *Appendix*, a simple relationship can be found between the different measured fluxes to obtain the water flux (*i*) through the endogenous water permeability (J_{ENDO}), (*ii*) through the passive SGLT1 water permeability (J_{SGLT1}), and (*iii*) through the water flux actually cotransported with Na^+ and αMG (J_{COTR}).

Eleven experiments of the type shown in Fig. 2 were performed. A rapid inhibition of the cotransport current by addition of 100 μM Pz produced an immediate reduction in this swelling rate due to the reduction in membrane water permeability from L_P^{SGLT1} to $L_P^{SGLT1+Pz}$. As the water flux was observed to continue to decrease at a slower rate in the following several minutes, the initial swelling rate immediately after Pz addition was accurately

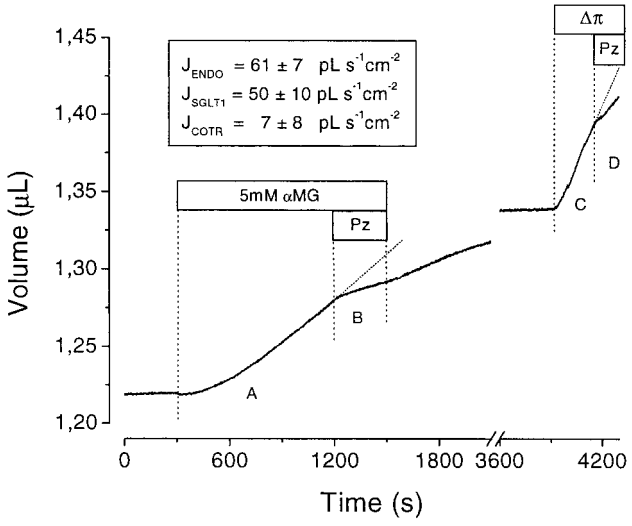

Fig. 2. Analysis of water transport during prolonged exposure to 5 mM αMG. Oocyte volume is displayed as a function of time. αMG was present between $t = 300$ s and 1,500 s, and Pz was added as indicated. After a recovery period in the absence of αMG, an osmotic shock was imposed to enable water permeability measurement. (*Inset*) Calculated fluxes passing through the three presumed water pathways: endogenous water permeability (J_{ENDO}), SGLT1 passive permeability (J_{SGLT1}), and cotransported with Na^+ and αMG (J_{COTR}).

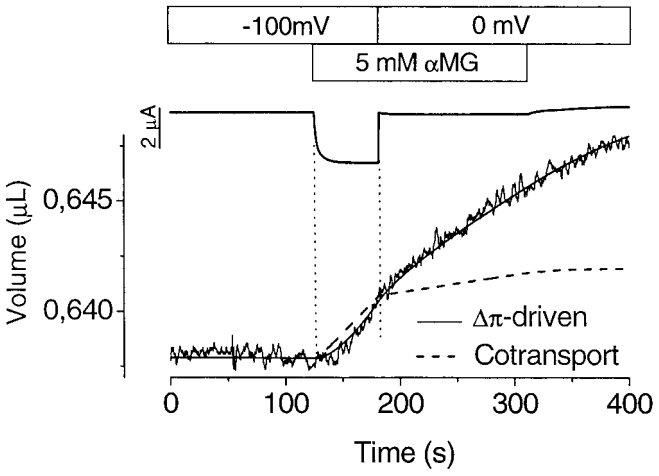

Fig. 3. Effect of a rapid reduction in the cotransported current. After a stabilization period at a membrane potential of −100 mV, 5 mM αMG is introduced in the bathing solution. After establishment of a steady-state cotransport current (a 60-s period), the membrane potential was stepped to 0 mV to abruptly reduce the cotransport current. At $t = 300$ s, αMG was removed to allow a determination of background current at 0 mV. The volume data were either fitted according to the water-cotransport hypothesis (240 water molecules/αMG molecule, dashed line) or by using the osmolyte accumulation model (full line).

estimated by the value of $(\Delta V/\tau)$ by using the following equation to fit the volume vs. time curve.

$$V = V_{ini} + \Delta V(1 - e^{-(t - t_{ini})/\tau}), \qquad [3]$$

where V_{ini} and t_{ini} are the volume and time when Pz was added, and τ is the time constant associated with the additional volume (ΔV) that would be gained during a prolonged transport inhibition period. In the presence of Pz, $L_P^{SGLT1+Pz}$ averaged 2.5 ± 0.2 × 10^{-4} cm·s^{-1}, and the initial cell swelling right after Pz addition was 61 ± 7 pL·s^{-1}·cm^{-2}. Individual values of these two parameters were used to estimate the effective osmotic gradient ($\Delta\pi$) for each of the 11 experiments, which yielded an average of 16 ± 2 mOsm. In the absence of Pz, L_P^{SGLT1} averaged 4.0 ± 0.3 × 10^{-4} cm·s^{-1}, and the individual values of L_P^{SGLT1} with their corresponding $\Delta\pi$ were used to calculate an amplitude for the passive cell swelling of 110 ± 20 pL·s^{-1}·cm^{-2}. Using the equation presented in the *Appendix*, the water flux observed after 15-min exposure to αMG can be accounted as follows: 61 ± 7 pL·s^{-1}·cm^{-2} was attributed to J_{ENDO}, 50 ± 10 pL·s^{-1}·cm^{-2} was attributed to J_{SGLT1}, and only 7 ± 8 pL·s^{-1}·cm^{-2} can be associated with a putative water cotransport process (not statistically different from 0, $P = 0.40$). It is concluded from the present series of 11 experiments that, after a 15-min exposure to αMG, a significant amount of osmolyte is accumulated under the oocyte membrane and that this transmembrane osmotic gradient is sufficient to drive water flux through passive pathways, which can quantitatively account for the total cell swelling observed.

Absence of early water cotransport. Because it may be difficult to detect a relatively small J_{COTR} in the presence of a large transport-related osmotic gradient, the presence of J_{COTR} was tested in the first minute after the activation of a cotransport current. As αMG addition to the bath takes ≈20 s, we chose to modulate the cotransport activity by changing the membrane potential from −100 mV to 0 mV, a procedure that takes only 10 ms with our system. The time course of the oocyte volume, as predicted from the water cotransport hypothesis (V_{COTR}), should follow the integrated current, as:

$$V_{COTR} = V_0 + \frac{N\bar{v}_W}{2F}\int_0^t I_{COTR}\cdot dt, \qquad [4]$$

where V_0 is the initial oocyte volume, N is the number of water molecules per αMG transported, F is the Faraday constant, and I_{COTR} is the αMG-dependent cotransport current. In this series of six experiments, the oocyte was maintained at −100 mV until the volume reached a stable level. At this time, 5 mM αMG was presented in the bath and after 60 s the holding potential was stepped to 0 mV (see Fig. 3). Upon depolarization, the cotransport current decreased abruptly by 94 ± 1% but the oocyte swelling rate was reduced by only 25 ± 2%, as measured in a 20-s period after the voltage step. The theoretical prediction (Eq. **4**) is shown in Fig. 3 by using a constant N that matches the water flux observed at −100 mV ($n = 240$ water molecules per αMG transported in the case presented). It will be shown below that a model based on transport-dependent intracellular accumulation of osmolytes can precisely account for the cell volume time course presented in Fig. 3.

Role of Cations in Osmolyte Accumulation. Accumulation of transport-dependent osmolytes must involve αMG as well as a certain amount of Na^+ accompanied by some intracellular anion to obey the electroneutrality law (global electroneutrality being taken care of by the current electrode of the voltage clamp system). Indeed, the osmotic effect of a cation transport is not trivial as one Na^+ ion entering the cell can either attract an intracellular anion or repel an intracellular cation, generating a local accumulation of two osmolytes in the first case but none in the second (if the attracted or repelled ion is monovalent). To determine the osmotic importance of the cation during Na^+/αMG cotransport, the cell swelling produced by the generation of a purely cationic current was measured in six oocytes expressing the potassium channel ROMK2. The osmolality gradient caused by K^+ uptake can be estimated from the rate of cellular swelling and the reversal potential of the K^+ current (E_K) can be used to obtain an independent estimation of the local intracellular K^+ concentration ($[K]_i$). Given the facts that the selectivity of ROMK2 for

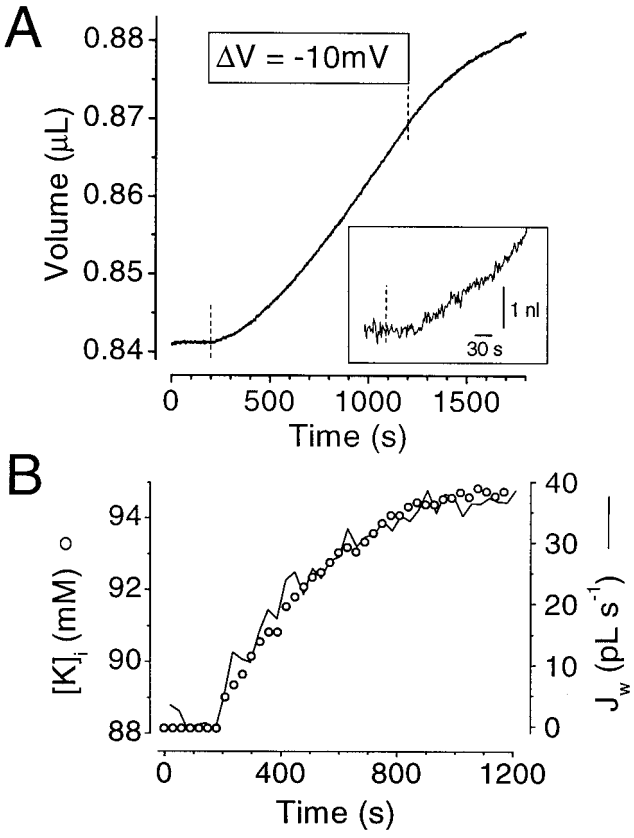

Fig. 4. Effect of a K^+ inward current on oocyte volume and intracellular K^+ concentration. (*A*) Average volume measurements in ROMK2-injected oocytes ($n = 3$). At $t = 180$ s, the oocyte membrane was hyperpolarized by 10 mV to stimulate an inward K current averaging 1.8 μA. (*Inset*) Enlargement of the initial portion of the curve shown in *A*. The dotted line represents the time at which the inward current was stimulated. (*B*) Parallel increases in intracellular K^+ concentration ($[K]_i$, ○) and in the instantaneous water flux (J_w, full line). $[K]_i$ was obtained from the K^+ reversal potential and J_w was obtained from the slope of the curve shown in *A*, which was measured every 30 s using a time window of ± 25 s.

K^+ is very high (17) and oocytes expressing the channel present a conductance 2 orders of magnitude larger than noninjected oocytes, the total oocyte current was assumed to represent a pure K^+ current. In this series of experiments, the volume of ROMK2-expressing oocytes was measured as the membrane potential was stepped from the reversal potential in the presence of 20 mM K^+ to a potential 10 mV more negative. The inward K^+ current averaged 1.8 ± 0.2 μA and triggered both a cell swelling and a significant increase in $[K]_i$ averaging 8 ± 2 mM in 10 min ($P < 0.01$, $n = 6$, from a baseline of 90 ± 5 mM). Fig. 4*A* shows the average time course of these effects from three synchronized experiments where E_K was measured every 30 s. The swelling rate, plotted in Fig. 4*B*, was obtained from the slope of the curve in Fig. 4*A*, which also was determined every 30 s. From Fig. 4, it is clear that an inward K^+ current can generate an accumulation of intracellular K^+ that can be readily detected at 30 s and that triggers a parallel cell swelling.

Computer Simulation. To understand and predict the osmolyte accumulation inside the oocyte, a theoretical model of diffusion was elaborated. An oocyte of a given radius is separated in 50 concentric shells of 10 μm thickness and a final inner sphere (of ≈100 μm in radius). The osmotic force driving water inside the oocyte is the osmotic gradient between the first oocyte shell and the external solution. As the osmolality of the external solution is considered constant, the goal of the model is to obtain the osmolality of the first shell allowing the calculation of J_w. In this model, a single average diffusion coefficient is used for the diffusion of αMG as well as for Na^+ and its accompanying anion. At each time increment (dt), an amount of solute ($J_1\,dt$) entering the oocyte first shell is obtained from the cotransport current as follows:

$$J_1 dt = \frac{n_{\alpha MG}}{2FS} I dt, \qquad [5]$$

where $n_{\alpha MG}$ is the number of osmolytes per αMG molecule transported. The intracellular solutes were allowed to diffuse inside the oocyte from shell i to shell $i + 1$, following Fick's first law:

$$J_{i,i+1} = D\frac{(C_i - C_{i+1})}{\Delta x}, \qquad [6]$$

where C_i and C_{i+1} are the added solute concentration in the i and $i + 1$ shells, respectively, Δx corresponds to 10 μm, and D is the intracellular diffusion coefficient. Changes in C_i were calculated by using Eqs. **5** and **6,** taking into account the fact that the water content of an oocyte was found, in preliminary experiments, to be 85% of its geometric volume (data not shown). Finally, at each time increment, the volume of the first shell was allowed to increase due to the water uptake driven by the local hypertonicity developed. The oocyte theoretical volume at a time t [$V(t)$] is given by:

$$V(t) = V(t - 1) + \bar{v}_W L_P S C_1 dt, \qquad [7]$$

where $V(t - 1)$ is the oocyte total volume calculated at the previous time increment. The value of 10 μm for the shell thickness was chosen to yield a satisfactory resolution in the C_i vs. distance curves and, with this shell thickness, the selected time increment could not exceed 0.1 s without generating instabilities in the determination of $C_i(t)$.

In a series of five experiments, the model was used to reproduce the time course of the oocyte volume as αMG was added for 15 min followed by a recovery period of similar duration. Basically, only two parameters needed to be adjusted: the intracellular diffusion coefficient D and $n_{\alpha MG}$ (the number of osmolytes accompanying one αMG molecule). In four of five instances, the value of L_P^{SGLT1} was precisely adjusted to account for the cell swelling induced by a 2-min hypotonic schock (−50 mOsm) performed at the end of the experimental period (see Fig. 5). In the remaining case, L_P^{SGLT1} was considered as an additional adjustable parameter. The average of intracellular diffusion coefficients used was 1.1 ± 0.3 × 10^{-6} $cm^2 \cdot s^{-1}$, and the average $n_{\alpha MG}$ was 4.1 ± 0.3 osmolytes per αMG molecule. Interestingly, as shown in Fig. 3, shorter time course experiments also could be fitted with the model using the average L_P^{SGLT1} and values for D and $n_{\alpha MG}$ that are within the range used for the long time course experiments.

Discussion

The suggestion that the Na^+/glucose cotransporter (SGLT1) can act as a water pump has generated a significant interest among transport physiologists (18–20) and appeared well supported experimentally (5, 8, 9). In the present study, crucial experiments demonstrate dissociation between the solute and water fluxes, which is inconsistent with the hypothesis of water cotransport but supports our contention that the water flux through SGLT1 is essentially passive, being driven by a readily detectable local osmotic gradient. In the following paragraphs, we will review our evidence and compare it to the corresponding evidence used to support the water cotransport hypothesis.

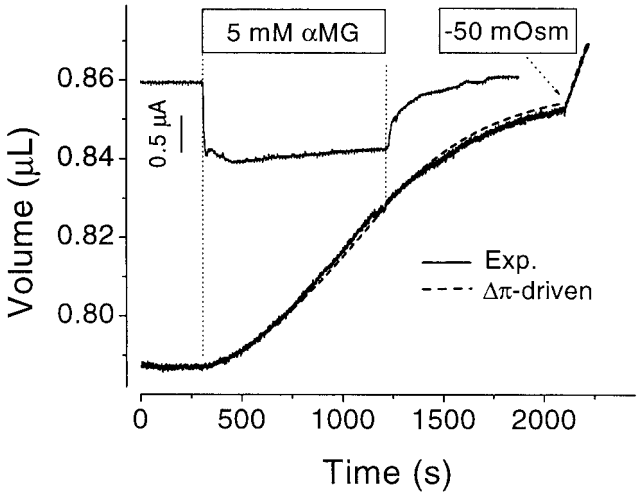

Fig. 5. Theoretical fit using the osmolyte accumulation model. Oocyte current and volume are shown as a function of time. From t = 300–1,200 s, 5 mM αMG was added to the bathing solution and at t = 2,100 s, 50 mM mannitol was removed from the external solution. Superposition of the experimental data (full line) to the theoretical prediction (dashed line) from the accumulation model are presented.

SGLT1 Passive Water Permeability. First of all, the present study confirms that water can be driven through SGLT1 as oocytes injected with SGLT1 cRNA display an additional water permeability that can be blocked by Pz, the specific inhibitor of SGLT1. In a series of 11 oocytes expressing an average of 800 nA of cotransport current, the additional water permeability for the whole oocyte was 0.64×10^{-4} cm^3·s^{-1} [i.e., $(L_P^{SGLT1} - L_P^{SGLT1+Pz}) \times S$]. Using a turnover rate estimated at 57 s^{-1} for the human SGLT1 (21), the average cotransport current would correspond to 4×10^{10} cotransporter molecules per oocyte, and the water permeability of individual cotransporters would be of the order of 1.5×10^{-15} cm^3·s^{-1}. In comparison, the single-channel water permeabilities of aquaporins 1, 2, 3, and 5 were reported as varying between 20 and 60×10^{-15} cm^3·s^{-1} (22), i.e., 10–40 times larger than the present estimate for SGLT1.

The Relationship Between Cotransport Current and Water Flux. The present study also confirms that isotonic addition of αMG generates a cell swelling (see Fig. 1) that can last for hours as reported (5, 8, 9). The absence of a significant time delay between glucose addition and cell swelling was a first criterion used in early studies to support the water cotransport hypothesis (8). In the present study, we didn't attempt to determine a time delay for two reasons: (*i*) accurate determination of oocyte volume requires a rather gentle solution flow rate and the time required to reach 80% of the cotransport current is already of the order of 15–20 s (ref. 8 and present study), and (*ii*) the extrapolation normally used to determine a time delay is inaccurate and subjective and yields a wide range of time delays that often include negative values that are clearly artifactual.

A better way of looking at the relationship between cotransport current and water flux is to measure oocyte volume while the cotransport current is changed by a rapid step in membrane potential. This series of experiments (see Fig. 3) clearly shows that the cell swelling does not follow the amount of αMG transported (i.e., the integrated cotransport current) as predicted by the water cotransport hypothesis. This is in contradiction with previously published results where an immediate effect of an hyperpolarization from 0 to −100 mV was reported (5). We think that the experimental protocol used in the present study is more discriminant than the protocol previously used because (*i*) enough time was spent at −100 mV to make sure that the swelling rate closely corresponds to the putative water cotransport (≈240 water molecules per αMG molecule) and (*ii*) using 5 mM αMG with the human SGLT1 provides for a dramatic reduction in the cotransport current as only 6% of the current measured at −100 mV remained at 0mV. In contrast, Loo *et al.* (5) used the rabbit SGLT1 for which, in their experimental conditions, the cotransport current measured at 0 mV is as much as 29% of the current measured at −100 mV. In addition, their result is difficult to interpret as the experiment was performed in the presence of an osmotic gradient (the external solution being hypotonic by 15 mOsm), which generates an initial swelling rate at 0 mV that is, in fact, higher than could be expected from the water cotransport hypothesis. As shown in Fig. 3, the cell swelling induced by a strong cotransport current appears almost "irreversible" when the current is rapidly shut down, an observation in good agreement with the hypothesis of intracellular osmolyte accumulation.

Effect of a Pure K^+ Current (ROMK2) on Water Flux. A second criterion used to support the water cotransport hypothesis was the reported absence of cell swelling (in the first 30 s) caused by the stimulation of an ionic current using different ionophores or ionic channels (8, 9). In our series of experiments with oocytes expressing ROMK-2, it was found that a K^+ influx is fully capable of producing a cell swelling on a long-term basis (≈5 min) that is smaller in amplitude but qualitatively similar to what could be observed with a cotransport current (compare Figs. 1 and 4). Measurements of E_K show that the intracellular K concentration increases by 8 mM in 10 min, and this increase is already detectable 30 s after initiating the K^+ inward current. Not surprisingly, the full time course of the K^+-induced swelling can be accurately reproduced with the osmolyte accumulation model. However, care must be taken in directly comparing the cotransport and the K^+ current experiments. First, SGLT1-expressing oocytes present a water permeability that is about twice the permeability of a noninjected oocyte. This makes SGLT1-expressing oocytes much more sensitive to any osmolyte accumulation than noninjected oocytes. Second, a Na/glucose cotransport current carries with it an extra osmolyte with respect to an equivalent K^+ current. This is important as, unlike αMG, injected cations do not simply accumulate under the oocyte membrane. Considering the electroneutrality law, a influx of cations across the oocyte membrane is matched by a compensatory charge displacement at the current-injecting electrode. Depending on the relative mobilities of intracellular cations and anions, osmolytes (or salt) accumulation will be divided between these two locations: under the oocyte membrane and around the current electrode. These two factors should increase the relative sensitivity of SGLT1-expressing oocytes vs. ROMK-2-expressing oocytes in the face of transport-related osmolyte accumulation.

Osmolyte Accumulation Model. The data presented in Fig. 2 clearly indicate the presence of a transport-dependent osmotic gradient after a prolonged stimulation of a cotransport current. We tested the possibility of accounting for the build-up of such an osmotic gradient by using the simplest diffusion model for SGLT1-expressing oocyte. As shown in Fig. 5, the rate of cell swelling when αMG is added and removed from the bath can be accurately accounted for by the adjustment of only two parameters: $n_{\alpha MG}$, the number of osmolyte accumulated per αMG molecule transported, and D, the average intracellular diffusion coefficient for the "average" intracellular osmolyte. The best value of $n_{\alpha MG}$ was found to be 4.1, which is in good agreement with the known stoichiometry of the cotransporter and a significant Cl^- injection by the KCl-filled current electrode. The best diffusion coefficient was 1.1×10^{-6} cm^2·s^{-1}, which is about five times lower than the diffusion coefficient expected for a small molecule or NaCl in Barth's solution. This is in qualitative agreement with a recent determination of the intracellular

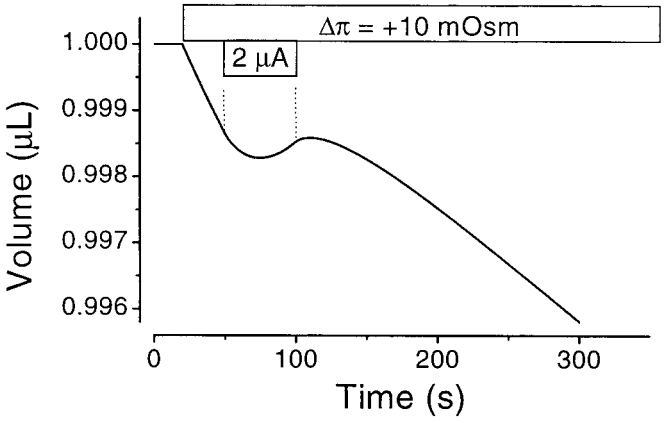

Fig. 6. Passive water transport against an apparent osmotic gradient. The osmolyte accumulation model was used to calculate the oocyte volume as a function of time after presenting an hypertonic (+10 mOsm) bath solution. The parameters used were: $L_p = 7 \times 10^{-4}$ cm·s^{-1}, $D = 0.5 \times 10^{-6}$ cm^2·s^{-1}, and $n = 5$ osmolytes/αMG transported. From $t = 50$–100 s, a 2 μA inward cotransport current is applied.

conductivity of an oocyte that was found to be five times smaller than the extracellular conductivity of a normal Barth's solution (23). A low intracellular diffusion coefficient is indeed expected given the relatively high viscosity of the cytosol and the fact that only a fraction of the intracellular space is available for diffusion.

Finally, our simple diffusion model can be used to explain the capacity of SGLT1 to induce a change in oocyte volume against an osmotic gradient that had been previously used as a third criterion supporting the water cotransport hypothesis (9). In Fig. 6, using the parameters found with SGLT1-expressing oocytes, the calculated volume of an oocyte is shown as a function of time after changing the osmolality of the bathing solution from 200 to 210 mOsm. While the oocyte volume is experiencing a steady-state decrease, an inward cotransport current of 2 μA is applied for 50 s. This is shown to be sufficient to reverse the oocyte shrinkage into a swelling phase apparently "against" an imposed osmotic gradient. This is to be expected if the osmolyte accumulation is capable of "locally" reversing the imposed osmotic gradient of 10 mOsm. Clearly, the direction of the water flux cannot be taken as an indication of secondary active water transport in this experimental situation.

Conclusion

On a short-term basis, rapid depolarization has failed to reveal the presence of any specific water cotransport. On a long-term basis (several minutes), which is the physiologically relevant situation, the presence of a trans-membrane osmotic gradient is certainly the dominant driving force for water influx and the portion corresponding to the putative water cotransport was not found to be significant. Using a simple model based on intracellular solute diffusion, the full time course (0–30 min) of transport-dependent water influx can be correctly accounted for with a reasonable intracellular diffusion coefficient. Although it is clear that SGLT1 and some other solute transporters are significantly permeable to water, it was found that water transport was passive and required, in all studied cases, the presence of an osmotic gradient to energize it.

Appendix

Taking into consideration the water cotransport hypothesis, the observed water flux (J_w) is composed of three different water fluxes across the oocyte membrane: (*i*) the passive osmotic flux using the endogenous water permeability (J_{ENDO}), (*ii*) the osmotic water flux through the SGLT1 (J_{SGLT1}), and (*iii*) the cotransported water flux through SGLT1 (J_{COTR}). In the experiment shown in Fig. 2, the J_{COTR} was calculated for each individual oocyte from the fluxes measured in four conditions. In condition A, αMG is cotransported and all of the different water fluxes are present. Thus, the total flux in this time period (J_A) is $J_{ENDO} + J_{SGLT1} + J_{COTR}$. In condition B (see Fig. 2), Pz was added, blocking αMG transport and reducing J_{SGLT1} and J_{COTR} to 0. Thus, the total flux in condition B (J_B) is simply J_{ENDO} itself. In condition C, the water flux was caused by an osmotic shock of 50 mOsm ($\Delta\pi'$). In this case, the total water flux (J_C) is mediated by the endogenous permeability and SGLT1, but cannot be actively transported by SGLT1 because αMG is not present. Thus, J_C is $J'_{ENDO} + J'_{SGLT1}$. Finally, in condition D, the addition of Pz during the osmotic shock blocked the passive water flux through SGLT1. Thus, the total water flux (J_D) is only J'_{ENDO}. With these four conditions, posing that $\Delta\pi$ is the transport-dependent osmotic gradient and using the permeability definition given in Eq. **2**, we find:

$$\begin{aligned} J_{COTR} &= J_A - J_{ENDO} - L_P^{SGLT1}\cdot\Delta\pi \\ &= J_A - J_c\frac{J_{ENDO}}{J'_{ENDO}} \\ &= J_A - J_C\frac{J_B}{J_D}. \end{aligned} \qquad \textbf{[A1]}$$

We express our gratitude to Dr. Jason Xu and Steve C. Hebert from Vanderbilt for the gift of the ROMK2 clone. We also thank Ms. Bernadettte Wallendorff for her excellent technical help in several experiments. This work was supported by Medical Research Council of Canada Grant MT-10580.

1. Finkelstein, A. (1987) *Water Movements Through Lipid Bilayers, Pores, and Plasma Membranes: Theory and Reality* (Wiley, New York).
2. Fischbarg, J., Kuang, K. Y., Vera, J. C., Arant S., Silverstein, S. C., Loike, J. & Rosen, O. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3244–3247.
3. Agre, P., Preston, G. M., Smith, B. L., Jung, J. S., Raina, S., Moon, C., Guggino, W. B. & Nielsen, S. (1993) *Am. J. Physiol.* **265,** F463–F476.
4. Loike, J. D., Hickman, S., Kuang, K., Xu, M., Cao, L., Vera, J. C., Silverstein, S. C. & Fischbarg, J. (1996) *Am. J. Physiol.* **271,** C1774–C1779.
5. Loo, D. D. F., Zeuthen, T., Chandy, G. & Wright, E. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13367–13370.
6. Diamond, J. M. & Bossert, W. H. (1967) *J. Gen. Physiol.* **50,** 2061–2083.
7. Loo, D. D. F., Hirayama, B. A., Meinild, A. K., Chandy, G., Zeuthen, T. & Wright, E. M. (1999) *J. Physiol. (London)* **518,** 195–202.
8. Zeuthen, T., Meinild, A. K., Klaerke, D. A., Loo, D. D. F., Wright, E. M., Belhage, B. & Litman, T. (1997) *Biol. Cell.* **89,** 307–312.
9. Meinild, A. K., Klaerke, D. A., Loo, D. D., Wright, E. M. & Zeuthen, T. (1998) *J. Physiol. (London)* **508,** 15–21.
10. Chen, X. Z., Coady, M. J., Jackson, F., Berteloot, A. & Lapointe, J. Y. (1995) *Biophys. J.* **69,** 2405–2414.
11. Chen, X. Z., Coady, M. J., Jalal, F., Wallendorff, B. & Lapointe, J. Y. (1997) *Biophys. J.* **73,** 2503–2510.
12. Boim, M. A., Ho, K., Shuck, M. E., Bienkowski, M. J., Block, J. H., Slightom, J. L., Yang, Y., Brenner, B. M. & Hebert, S.C. (1995) *Am. J. Physiol.* **268,** F1132–F1140.
13. Coady, M. J., Jalal, F., Chen, X., Lemay, G., Berteloot, A. & Lapointe, J. Y. (1994) *FEBS Lett.* **356,** 174–178.
14. Bissonnette, P., Noël, J., Coady, M. J. & Lapointe, J. Y. (1999) *J Physiol. (London)* **520,** 359–371.
15. Schreibmayer, W., Lester, H. A. L. & Dascal, N. (1994) *Pflügers Arch.* **426,** 453–458.
16. Zampighi, G. A., Kreman, M., Boorer, K. J., Loo, D. D. F., Bezanilla, F., Chandy, G., Hall, J. E. & Wright, E. M. (1995) *J. Membr. Biol.* **148,** 65–78.
17. Zhou, H., Tate, S. S. & Palmer, L. G. (1994) *Am. J. Physiol.* **266,** C809–C824.
18. Diamond, J. M. (1996) *Nature (London)* **384,** 611–612.
19. Reuss, L. (1996) *J. Physiol. (London)* **497,** 1.
20. Spring, K. R. (1999) *News Physiol. Sci.* **14,** 92–100.
21. Panayotova-Heiermann, M., Loo, D. D. F. & Wright, E. M. (1995) *J. Biol. Chem.* **270,** 27099–27105.
22. Zeuthen, T. & Klaerke, D. A. (1999) *J. Biol. Chem.* **274,** 21631–21636.
23. Baumgartner, W., Islas, L. & Sigworth, F. J. (1999) *Biophys.* **77,** 1980–1991.