J. Membrane Biol. 143, 189–197 (1995)

The Journal of
Membrane
Biology

The Proximal Straight Tubule (PST) Basolateral Cell Membrane Water Channel: Selectivity Characteristics

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Received: 16 May 1994/Revised: 15 August 1994

Abstract. Proximal straight tubules (PST) were dissected from rabbit kidneys, held by crimping pipettes in a chamber and bathed in a buffered isosmotic (295 mOsm/kg) solution containing 200 mM mannitol (MBS). Changes in tubule diameter were monitored on line with an inverted microscope, TV camera and image processor. The PST were then challenged for 20 sec with MBS made 35 mOsm/kg hyperosmotic by addition of either NaCl, KCl, mannitol (M), glycerol (G), ethylene glycol (E), glycine (g), urea (U), acetamide (A) or formamide (F). With NaCl, KCl, M, G, E, g, U, and A, tubules shrunk osmometrically within 0.5 sec and remained shrunk for as long as 20 sec without recovering their original volume (sometimes A showed some recovery). PST barely shrunk with F and quickly recovered their original volume. The permeability coefficients were 0 μm/sec (NaCl, M, g, E and U), 1 μm/sec (A), 84 μm/sec (F) and 0.02 μm/sec (G). The reflection coefficients $\sigma = 1.0$ (NaCl, KCl, M, G, E, g and U), 0.95 (A) and 0.62 (F). Similar σ values were obtained by substituting 200 mOsm/kg M in MBS by either NaCl, KCl, G, E, g, U, a or F. The olive oil/water partition coefficients are 5 (M), 15 (U), 85 (A) and 75 (F) (all $\times 10^{-5}$). Thus, part of F permeates the cell membrane through the lipid bilayer. The probing molecules van der Waals diameters are $7.4 \times 8.2 \times 12.0$ (M), $3.6 \times 5.2 \times 5.4$ (U), $3.8 \times 5.2 \times 5.4$ (A) and $(3.4 \times 4.5 \times 5.4$ (F) Å. We conclude that only F clearly permeates the water channel (WCH). Water molecules must single file within the WCH. After subtraction of the bilayer permeability of the probes, we estimate for the WCH selectivity filter cross-section a diameter of 4.2—4.7 Å (if it is circular) and 3.6×4.2 Å (if it is rectangular). But if the oxygens facing the WCH lumen H bond with the molecules crossing the WCH, the WCH selectivity filter would be 3.3–3.8 Å (circular) and 3.6×4.0 Å (rectangular).

Key words: Water pore sizes — Water channels — Reflection coefficients — Selectivity filters — Proximal kidney tubule — Single file transport

Introduction

Full understanding of water absorption across the proximal tubule (be it transcellular or paracellular or both, Whittembury, Carpi-Medina & Gonzalez, 1987; Carpi-Medina & Whittembury, 1988; Schafer, 1990; Schafer, Reeves & Andreoli, 1992; Verkman, 1989; Whittembury & Reuss, 1992) and volume regulation (i.e., water movement between cytosol and extracellular fluid across the cell membrane of proximal tubule cells) requires detailed knowledge of the water pathways across the cell membrane of this nephron segment (Whittembury et al., 1993). This paper examines some biophysical characteristics of the water pathways across the basolateral cell membrane of the proximal straight tubule of the rabbit kidney. Previous observations (Whittembury et al., 1984; Whittembury & Carpi-Medina, 1988; Verkman, 1989; Verkman, van Hoeck & Zhang, 1993) indicated that the water osmotic (net) permeability (P_{os}) is 10–18 times larger than the water diffusive (isotope) permeability (P_d) (Carpi-Medina et al., 1988). The Arrhenius energies of activation (E_a) of both permeabilities were ≈4 kcal/mol as expected from free water movement, indicating a low degree of interaction between the water molecules moving across the cell membrane and the water pathway. Addition of the sulfhydryl reagent *para*-chloromercuribenzene sulfonic acid (pCMBS) curtailed P_{os} to ≈10% of the control value and also P_d. Under these circumstances, the P_{os}/P_d ratio was 2–3 and the values for E_a of both permeabilities were 9–10 kcal/mol.

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Thus, the interaction between membrane and the water molecules crossing it increased to values similar to those obtained in pure lipid bilayers without channels (Fettiplace & Haydon, 1980; Finkelstein, 1987, 1993). These results were interpreted to indicate that under the control conditions ≈90% of the net water movement occurred through proteinic channels which were then rendered water-impermeable by the mercurial reagent. The remaining 10% of the water permeability indicated flow through the lipid bilayer. The action of pCMBS on the water permeability could be readily reversed by addition of dithiothreitol (Whittembury et al., 1984). However, these results do not differentiate whether water flows within the channels in a single file or whether the water channels are wide (Heckmann, 1972; Finkelstein, 1987, 1993; Whittembury & Carpi-Medina, 1988).

Recent studies show that cell membrane diffusive permeability to urea can be inhibited under conditions in which the water permeability is unaffected (Echevarría, 1990; Echevarría et al., 1994) indicating that in proximal tubule cells the water channel does not let urea through. Thus, water molecules must single file within its lumen with a selectivity filter internal diameter between 3.0 Å (the diameter of the water molecule) and 4.5 Å (just below the diameter of the urea molecule) so that urea can be excluded from the channel.

In the present work we have used an independent approach. We have studied the interaction between water and probing molecules of graded molecular dimensions, to ascertain which of them interact with the water molecule within the water channel. For this purpose, the reflection coefficient σ and the permeability across the cell membrane of those probes were measured. The results lead to the conclusion that only formamide clearly interacts with water within the channel (its value of σ = 0.62 with one method and 0.61 with another). Part of the formamide crosses the lipid bilayer part of the membrane. The dimensions of the water channel selectivity filter (through which water single files) depend on whether its cross-section (Hille, 1992) is taken to be circular or rectangular and on whether H bonds form between oxygens facing the channel lumen and the molecules crossing the channel, but its dimensions must lie between 3.3 and 4.7 Å. Part of this research has been published in a preliminary form (Whittembury et al., 1991, 1992, 1993).

Materials and Methods

The method used to handle the tubules and to measure the water osmotic permeability (P_{os}) has been described in detail before (González et al., 1982; Carpi-Medina, González & Whittembury, 1983; Carpi-Medina et al., 1984; Whittembury et al., 1984, 1986). Briefly, New Zealand rabbits weighing 1.5–2 kg were used. Individual PST segments (about 0.5 mm in length) were dissected out of the upper kidney cortex. They were crimped with micropipettes in a chamber leaving a length of 0.2–0.3 mm of usable free tubular tissue between the pipettes, and then were kept in an isosmotic artificial bathing solution (ABS) which was equilibrated with 95% O_2/5% CO_2 previously thermostated at 25°C. An inverted microscope was focused on the tubular axis using a 40× objective. The tubular image was recorded with a video camera. The video signal was analyzed with a real-time area-tracker (Lindemann, 1984; Carpi-Medina et al., 1984) which generates on line a signal proportional to the outer tubular diameter to an oscilloscope. Following the records, tubular volume was calculated as a function of time. The time resolution was 16.7 msec and the space resolution was near 0.03 μm (Whittembury et al., 1986). For the osmotic steps required to measure P_{os}, the chamber was quickly emptied and the chosen anisosmotic test medium was 95% replaced about the tubule in less than 100 msec, thus minimizing the effect of unstirred layers (Carpi-Medina et al., 1983; Pedley, 1983). When the diameter change was stabilized, the ABS was placed back into the chamber using the same procedure. During all these procedures tubule diameter was monitored as just described.

Bathing Solutions

All solutions were made up from a basic solution containing (in mM): KCl, 4.8; $NaHCO_3$, 25.0; $MgSO_4$, 1.2; $CaCl_2$, 1.9; Na_2HPO_4, 4.0; glucose, 8.3; alanine, 5.0, and the amount of NaCl required to achieve an osmolality of 95 mOsm/kg. Bovine serum albumin (6 g/dl) was added to this solution. NaCl (108 mM) was further added to the basic solution to achieve an osmolality of 295 mOsm/kg which is isosmotic to the tubules. This ABS was used to dissect and to equilibrate the tubules. Mannitol (200 mOsm/kg) added to the basic solution is also isosmotic to the tubules. This isosmotic bathing solution (295 mOsm/kg) with mannitol is denoted MBS. Dextran M_r 60,000 (1 mM), was used instead of albumin to prepare the *para*-chloromercuribenzene sulfonic acid (pCMBS)-containing solutions (at a concentration of 2 mM) to avoid reaction between this agent and the disulfide bonds of albumin (Whittembury et al., 1984). Osmolality differences across the peritubular aspect of the cells were achieved by using solutions with different concentrations of mannitol, NaCl or of other solutes which were used as probing molecules. The osmolality of the solutions was checked by freezing-point determination. All chemicals were obtained from Sigma Chemical.

Calculation of P_{os}

When the tubule is placed in a hyperosmotic medium made up with an impermeant solute (with a reflection coefficient σ = 1.0), the cell volume decreases as expected for a perfect osmometer within fractions of a second and remains shrunk (for a long as 20 sec) (González et al., 1982). The time course of the volume decrease (or of the volume increase if the new medium is hyposmotic) is used to calculate P_{os}, as has been described in detail previously (González et al., 1982; Carpi-Medina et al., 1983; Carpi-Medina et al., 1984). Briefly, as the tubule lumen was collapsed, only water flow through the basolateral membrane contributed to the volume change. Water flow from cell to bath or from bath to cell is provoked by a step change of the osmolality difference across the cell membrane, $\Delta C_o = C_c - C_{out}$, at time zero. This is achieved by increasing or decreasing C_{out}, the concentration of impermeant solutes in the bath. C_c is the cellular concentration of osmotically active substances. Cell volume changes are followed as diameter changes. Cell volume, V_c, and C_c will change with time. P_{os} may be obtained from Eq. (1):

$$P_{os} = [V_c^{iso}/(A_c \cdot C_{out} \cdot t)][1 - R + (b - K) \ln |(K - R)/(K - 1)|] \quad (1)$$

where A_c is the membrane area, $R = V_c/V_c^{iso}$, the ratio of tubule volume at times t and 0, respectively, and $K = (C_c^{iso}/C_{out})\ (1 - b) + b$, where C_c^{iso}/C_{out} is the ratio of external concentration before and after the solution change. Before the osmotic experiment begins $C_c^{iso} = C_{out}$, since the cells have an intracellular concentration isosmotic to that of the bath in which the tubule is kept. The cell solid content is b. Equation (1) was fitted to the data to calculate P_{os}.

Calculation of P_s

When the tubule is placed in a hyperosmotic medium made up with a permeant solute ($\sigma < 1.0$), the cell volume initially decreases and then returns towards its initial volume after passing through a well-defined minimum. At this minimum, the inward volume flow of the solute is balanced by the outward volume flow of water. Subsequently, the osmotic pressure gradient reverses its direction, in part because the impermeant solute is now more concentrated in the cell than in the medium. Water enters the cell along with the permeant solute. Based on this phenomenon Sha'afi et al. (1970) developed for red cells the minimum volume method to calculate the solute permeability P_s. We have used this method to calculate P_s across the basolateral aspect of PST cell membranes, using Eq. (2):

$$P_s = [R \cdot T \cdot V^w_{c\text{-min}} \cdot (d^2V^w_c/dt^2)_{min}]/[A_c^2 \cdot P_{os} \cdot \Delta C_o] \quad (2)$$

where $V^w_{c\text{-min}}$ is the volume of cell water at the minimum point. Independent experiments showed that within 20 sec of observation no noticeable volume regulatory phenomena were observed with the small osmotic gradients of impermeant solutes used here.

Equivalent Pore Diameter Calculation

It is accepted (Pappenheimer, Renkin & Borrero, 1951; Solomon, 1968, 1989; House, 1974; Renkin & Curry, 1979; Hill & Shachar-Hill, 1993) that for a cylindrical pore, the ratio of the restricted pore area for solute filtration (A_{sf}) to that for water filtration (A_{wf}) is a function of α and of α'. Where $\alpha = a/d$, and $\alpha' = w/d$. a, w, and d are the diameters of the molecule penetrating the pore, of the water molecule, and of the pore, respectively. Therefore, d can be calculated knowing A_{sf}/A_{wf} and a. The reflection coefficient σ may be defined (Dainty & Ginzburg, 1963; Kedem & Katchalsky, 1961, 1963; House, 1974) as:

$$\sigma = 1 - (A_{sf}/A_{wf}) - (P_s \cdot V_s/P_{os} \cdot V_w) \quad (3)$$

where V_s and V_w are the partial molar volumes of solute and water, respectively. In this equation the contributions to σ are: A_{sf}/A_{wf}, due to the solute-water frictional interaction in the channels; and $P_s \cdot V_s/P_{os} \cdot V_w$ if solute and water move through the membrane by separate pathways. Therefore, $\sigma = 1 - (A_{sf}/A_{wf})$ if the solute crosses the membrane exclusively through the water channels; $\sigma = 1 - (P_s \cdot V_s/P_{os} \cdot V_w)$ if the solute crosses the membrane exclusively through the lipid bilayer; and $\sigma < 1 - (P_s \cdot V_s/P_{os} \cdot V_w)$ if the solute crosses the membrane through the lipid bilayer and the water channels.

Measurements of σ

In the absence of transmembrane hydrostatic pressure differences the volume flow across the membrane will be zero when

$$\Delta C^i = -\sigma \cdot \Delta C^p \quad (4)$$

where ΔC^i and ΔC^p are the transmembrane concentration of impermeant and permeant solutes, respectively. Equation (4) allows the measurement of σ. The appropriate value of ΔC^p for a known ΔC^i (at zero net volume flow) was found by interpolation after performing a number of experiments which involved tubule cell swelling and tubule cell shrinking at different values of ΔC^p (Kedem & Katchalsky, 1958; Whittembury, Sugino & Solomon, 1960; Solomon, 1968; House, 1974).

The equivalent channel diameter can be assessed solving Eq. (3) for (A_{sf}/A_{wf}) with the values of P_{os}, P_s, and σ. The probes molecular dimensions were measured on Stuart and Brieglev models, except for raffinose taken from viscosity measurements (Whittembury et al., 1960; Schultz & Solomon, 1961).

Results

After 5–10 min equilibration in the MBS, the latter was substituted at time 0 (within 100 msec) by a hyposmotic solution in which 35 mM (or mOsm/kg) mannitol had been deleted. The osmolality of this solution was 260 mOsm/kg (made up by 95 mOsm/kg of the basic solution plus 165 mM (or mOsm/kg) mannitol. Cells swelled osmometrically within a second and equilibrated to their new volume. After 20 sec the 295 mOsm/kg MBS was placed back into the chamber. Cells shrunk osmometrically within a second and kept a volume close to their original one. Then MBS was substituted by a (330 mOsm/kg) hyperosmotic solution made up by addition of 35 mOsm/kg mannitol (to the MBS). Cells shrunk. After 20 sec the MBS was placed back, again within 100 msec. The tubules recovered their original volume. Alternatively, the osmolality of the solution was changed (at time 0) from 260 to 330 and *vice versa* and then to 295 mOsm/kg. The experiments were completely reversible; otherwise they were discarded as this indicated cell damage which could be also seen from the continuous microscopic observation. The initial rates of swelling and shrinking led to values of σ and P_{os}.

Measurements of σ

From the initial rates of swelling and shrinking $(dV_c/Adt)_{t\to 0}$, the mannitol concentration (ΔC^i) at which $(dV_c/Adt)_{t\to 0} = 0$, (interpolation by regression) was 35.0 ± 0.1 mOsm/kg (40 experiments) as referred to the 260 mOsm/kg solution (Fig. 1). In similar experiments either NaCl, KCl, urea, glycerol, ethylene glycol, or glycine was alternated with mannitol. With any of these solutes (20 experiments with each one), the concentration (ΔC^p) at which $(dV_c/Adt)_{t\to 0} = 0$ was not statistically different from 35 mOsm/kg (as referred to the 260 mOsm/kg solution). Their σ values calculated using Eq. (4) were not different from 1.00 (Table 1). Twenty experiments were also performed with acetamide. In 13 ΔC^p was 35 mOsm/kg and in 7, 41 mOsm/kg (as referred to the 260 mOsm/kg solution). As there was no reason to discard any of these values since the tubules behaved towards the

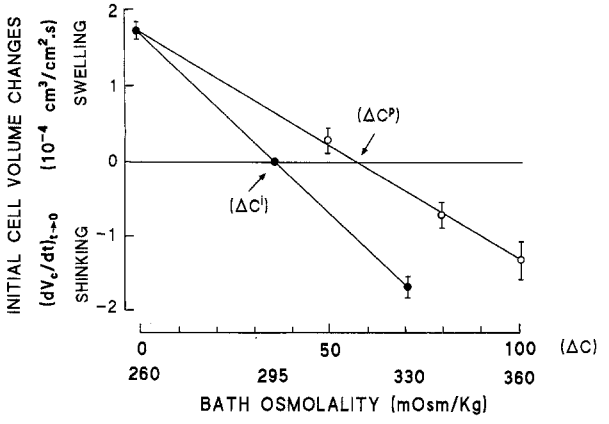

Fig. 1. Initial PST cell volume changes $(dV_c/Adt)_{t\to 0}$ (mean ± SEM) as a function of osmotic steps induced with mannitol (filled circles) or formamide (open circles). ΔC shows osmotic differences referred to the 260 mOsm/kg solution (made up by the 95 mOsm/kg basic solution plus 165 mM mannitol). Total bath osmolality is also shown in the abscissa. Solutions with higher osmolalities are made up of the 260 mOsm/kg solution plus either mannitol or formamide. Arrows point at $(dV_c/Adt)_{t\to 0} = 0$, for mannitol (ΔC^i) and formamide (ΔC^p).

Table 1. Reflection coefficients (σ) for various solutes

Solute	σ
Mannitol	1.00 ± 0.01 (40)
NaCl	1.05 ± 0.02 (20)
KCl	1.02 ± 0.03 (20)
Glycerol	0.99 ± 0.03 (20)
Ethylene glycol	1.01 ± 0.02 (20)
Glycine	0.99 ± 0.03 (20)
Urea	0.98 ± 0.03 (20)
Acetamide	0.95 ± 0.02 (20)
Formamide	0.62 ± 0.05 (20)

The number of experiments is given in parentheses.

other solutes homogeneously, the results have been grouped together. For acetamide σ = 0.95 (Table 1). An example of this behavior with acetamide is shown in Fig. 2, where submitting the tubule at time 0 to a 35 mOsm/kg hyperosmotic solution prepared by addition of 35 mOsm/kg acetamide to MBS resulted in 13 experiments in a response similar to that achieved with mannitol, glycerol, ethylene glycol, NaCl or urea, while in seven others resulted in a shrinking curve that passes through a minimum and then showed a tendency to slowly return towards the original isosmotic volume. From these curves a value of P_s for acetamide of 1 μm/sec was calculated (*see below* and Table 2).

In the 20 experiments with formamide PST behaved differently. PST shrunk slightly when exposed to the 35 mOsm/kg formamide hyperosmotic solution (35 mOsm/kg formamide added to MBS) and then recovered their original volume (Fig. 2). A 150 mOsm/kg formamide

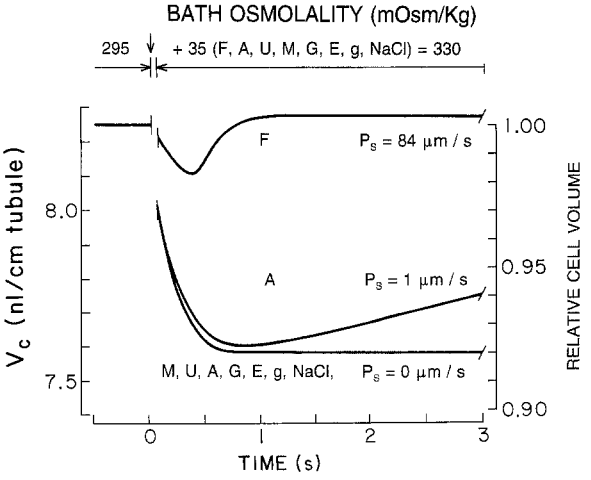

Fig. 2. Time course of tubule (cell) volume (V_c) after application at time = 0 of a (35 mOsm/kg) hyperosmotic step with various solutes. Tubules had been equilibrated in a 295 mOsm/kg MBS. At time = 0, MBS was replaced by a 35 mOsm/kg hyperosmotic solution prepared by addition (to MBS) of either formamide (*F*), acetamide (*A*), urea (*U*), mannitol (*M*), glycerol (*G*), ethylene glycol (*E*), glycine (*g*) or NaCl. The left- and right-hand side ordinates show cell volume (nl/cm tubule length), and relative cell volume, respectively. Curve *F* is the average of 20 experiments, the middle curve (*A*) is the average of 7 experiments, the lower one labeled (A) represents 13 experiments. It is identical to curves obtained with either M, U, G, E, g or NaCl (20 experiments each).

Table 2. Permeabilities P_s for various solutes

Solute	P_s (μm/sec)
Mannitol	0.00 (20)
NaCl	0.00 (10)
KCl	0.00 (10)
Glycerol	0.02 ± 0.05 (10)
Ethylene glycol	0.00 (10)
Glycine	0.00 (10)
Urea	0.00 (10)
Acetamide	1.00 ± 0.15 (20)
Formamide	84 ± 4 (20)

Measured with the minimum volume method (*see* Materials and Methods). The number of experiments is given in parentheses.

hyperosmotic solution (150 mOsm/kg formamide added to MBS), produced cell shrinking from an average 8.2 to 7.7 nl/cm. A minimum $(dV_c/Adt)_{t\to 0} = 0$ was reached from 350 msec after the solution replacement. Then the tubules showed some volume recovery reaching 8 nl/cm 1.2 sec after the solution change (*not shown* in Fig. 2). This indicated that (at a difference with mannitol, urea, NaCl, KCl, glycerol, ethylene glycol and urea) Formamide entered the cells through either the water channel or the lipid bilayer or both.

To measure $\sigma_{Formamide}$ (Fig. 1), PST were equilibrated in (295 mOsm/kg) MBS. Then MBS was substi-

tuted by a 260 mOsm/kg hyposmotic solution (95 mOsm/kg of the basic solution plus 165 mOsm/kg mannitol). Cells swelled. After 10 sec, MBS was placed back. Cells shrunk. After 10 sec, MBS was substituted by either a 310, 340, or 360 mOsm/kg formamide-containing solution (made up by the 260 mOsm/kg solution plus 50, 80, or 100 mM formamide, respectively) and 10 sec after the MBS was placed back. $(dV_c/Adt)_{t\to 0}$ values are plotted in Fig. 1. Interpolation by linear regression indicated that ΔC^p for formamide was 56.5 ± 0.05 mOsm/kg. Using Eq. (4), $\sigma_{Formamide}$ is (35.0/56.5) = 0.62 ± 0.05 (Table 1).

As mentioned in the Introduction, previous measurements of P_{os} (Whittembury et al., 1984; Carpi-Medina & Whittembury, 1988; Whittembury & Carpi-Medina, 1988) have disclosed that water crosses the basolateral PST cell membrane using two pathways: ≈90% through the water channels, which is inhibited by pCMBS. The remaining 10% (pCMBS-resistant P_{os}) represents water movement through the lipid bilayer part of the cell membrane. Formamide could be using either pathway or both. As mentioned in Materials and Methods (Eq. 3) penetration of formamide through the bilayer would result in a "low" $\sigma_{Formamide}$, due to $(P_s \cdot V_s/P_{os} \cdot V_w)$, which would not necessarily indicate that water and formamide shared the water channel. To differentiate between these possibilities, osmotic differences were set up across the basolateral PST cell membrane that would result in an inward flow of water. The inhibitory action of pCMBS on water inflow could then be used to help differentiate formamide penetration through the water channels from that through the bilayer. Five different experimental series were performed. All begin with equilibration of the PST in MBS (made up with the 95 mOsm/kg basic solution plus 200 mM mannitol). Then at time 0, MBS was replaced (within 100 msec) by the 95 mOsm/kg basic solution with various solutes added (one at a time) at an osmolality of 200 mOsm/kg or with no solute added (curve *X = 0* in Fig. 3). (i) The control series consisted of 6 groups of 10 experiments each. The added solute was either 200 mOsm/kg NaCl, KCl, urea, ethylene glycol, glycerol or glycine (dotted line *X = 200 NaCl, KCl, U, EG, G, g* in Fig. 3). No volume change was observed with any of these six solutes with a σ of 1.00. (ii) In 10 experiments the added solute was 200 mOsm/kg formamide (Fig. 3, curve *X = 200 F*). Although under these conditions there was also no osmotic difference across the cell membrane, cells swelled. $(dV_c/Adt)_{t\to 0}$ was 2.41 ± 0.35 (10^{-4} cm^3/cm^2 · sec). Under these conditions, formamide could be dragged by water through the water channel or it could be entering the cell through the lipid bilayer without interaction with water. (iii) A series of 12 experiments was similar to (ii) but in the presence of 2 mM pCMBS (Fig. 3, curve *X = 200 F + pCMBS*). The mean $(dV_c/Adt)_{t\to 0}$ was 0.31 ± 0.05 (10^{-4} cm^3/cm^2 · sec). Under these conditions, water should permeate only the bilayer. As most water channels should be closed, there should be no solvent drag of formamide. Therefore, the small but significant water inflow must be due to water crossing the cell membrane through the bilayer with formamide. If in series (ii) formamide were to cross the cell membrane exclusively through the bilayer and not by solvent drag, formamide penetration should be similar in experiments (ii) and (iii), as the difference in formamide concentration and in chemical activity of water is similar in both circumstances. Therefore, the difference between curves (200 F) and (200 F + pCMBS) in Fig. 3 indicates that in curve (200 F) formamide was interacting with water being dragged within the water channels. (iv) In 10 experiments, no solute was added to the basic solution (curve *X = 0*, Fig. 3). Thus, a 200 mOsm/kg difference (between C_c, the 295 mOsm/kg intracellular osmolytes and the basic solution) was set up across the cell membrane. Under these circumstances cells swelled at a mean initial rate $(dV_c/Adt)_{t\to 0}$ of 6.22 ± 0.75 (10^{-4} cm^3/cm^2 · sec) almost doubling tubule volume. After 20 sec the MBS was placed back (*not shown*). The PST recovered their original volume underscoring the reversibility

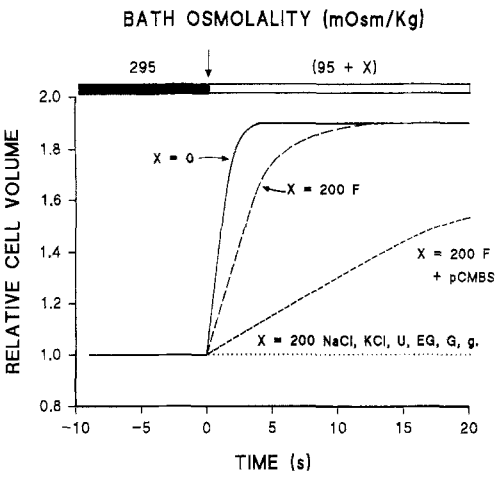

Fig. 3. Time course of relative cell volume changes after the application of different osmotic steps. The tubules had been equilibrated in 295 mOsm/kg MBS. At time 0, MBS is replaced (within 100 msec) by the 95 mOsm/kg basic solution plus X mOsm/kg of solutes. Results of four different experiments are shown. (i) The dotted line at the bottom (*X = 200*) is the average of 6 groups of 10 experiments each in which the added solute is 200 mOsm/kg of either NaCl, KCl, urea (*U*), ethylene glycol (*E*), glycerol (*G*) or glycine (*g*). There was no volume change. (ii) The line with the long dashes (*X = 200 F*) is the mean of 10 experiments in which the added solute is 200 mOsm/kg formamide. Cells swell. Initial mean rate, 2.41 μm/sec. (iii) The line with the short dashes (*X = 200 F + pCMBS*) is the mean of 12 experiments performed as in (ii) but with 2 mM pCMBS added to MBS (30 min before time 0) as well as to the formamide-containing solution. Cells swell. Initial mean rate, 0.31 μm/sec. (iv) The continuous line is the mean of 10 experiments. No solute was added to the basic solution (X = 0; i.e., 0 mOsm/kg of added osmolality). Cells swell. Initial mean rate, 6.22 μm/sec.

of this procedure. (v) Finally, 10 experiments were performed similar to (iv) but with 2 mM pCMBS to inhibit water channel activity. The rate of swelling (*not shown* in Fig. 3) dropped 15 times (compared to series iv) to a mean $(dV_c/Adt)_{t\to 0}$ of 0.43 ± 0.05 (10^{-4} cm^3/cm^2 · sec). This flow should reflect water permeation through the lipid bilayer due to the presence of the 200 mOsm/kg osmotic difference across the cell membrane (Whittembury et al., 1984; Whittembury & Carpi-Medina, 1988).

We are now in a position to reassess $\sigma_{Formamide}$ measurements (Table 1). The experiment of series (iii) shows that Formamide permeation through the bilayer part of the cell membrane is reflected in a volume flow inwards of 0.31 (10^{-4} cm^3/cm^2 · sec). If the value of σ for formamide were to be close to 1 the experiment in series (ii) should have resulted in a water inflow closer to 0.31. The observation that (in the absence of pCMBS) the inflow is 2.41 (10^{-4} cm^3/cm^2 · sec) agrees with a $\sigma_{Formamide}$ value of 0.62 ± 0.05. Thus, 200 mOsm/kg formamide retarded water inflow in 3.81 (10^{-4} cm^3/cm^2 · sec) from 6.22 (series iv, when the osmotic gradient is 200 mOsm/kg inwards) to 2.41 (series ii, when the osmotic gradient should be balanced by 200 mM formamide). The ratio 3.81/6.22 is 0.61 ± 0.11 (mean ± SEM).

Measurements of P_{os}

From the swelling and shrinking curves, the values of $dV_c/Adt)_{t=0}$ and the other parameters required in Eq. (1), the value of P_{os} was calculated by fitting this equation to the data. The values obtained with mannitol agreed with previous measurements (Whittembury et al., 1984; Carpi-Medina & Whittembury, 1988). Mean P_{os} was 46 ± 9.3 (10^{-4} cm^3/cm^2/sec.Osmolar, 40 experiments) equivalent to 2,553 ± 516 (10^{-4} cm/sec). This mean value corresponds in Figs. 1 and 2 to a slope $(dV_c/Adt)_{t=0}$ of 1.6 × 10^{-4} cm^3/cm^2 · sec, and in Fig. 2 to a change from 8.2 to 7.7 nl/cm/sec, for a tubule 32 μm in diameter in 290 msec with a hyperosmotic difference of 35 mOsm/kg.

Measurement of P_s

With curves as those shown in Fig. 2, P_s was calculated using Eq. (2). The results are shown in Table 2. The values of P_s for mannitol, NaCl, glycine, ethylene glycol and urea were 0. For acetamide an average value of 1 μm/sec was calculated; one of 84 μm/sec for formamide and 0.02 μm/sec for glycerol.

Calculation of A_{sf}/A_{wf}, and the Equivalent Pore Diameter

Using the values from Tables 1 and 2 and Eq. (3) the values for A_{sf}/A_{wf} for various probing molecules were calculated. They are shown in Fig. 4, plotted as a function of their average molecular diameters (Whittembury et al., 1960). The values are formamide, 0.16 ± 0.03; urea, 0.0; acetamide, 0.05 ± 0.02; ethylene glycol, glycerol and mannitol, 0.0.

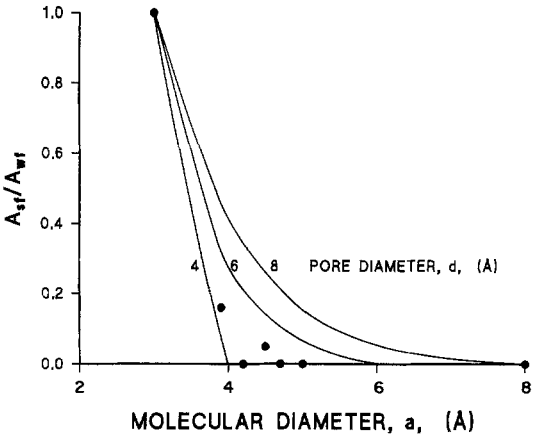

Fig. 4. Ratio of the area for restricted solute filtration (by convection) to that for water filtration (A_{sf}/A_{wf}), as a function of the diameter of water and of the probing molecules, a, in Å. From left to right in order of increasing diameters, the molecules are water, formamide, urea, acetamide, ethylene glycol, glycerol and mannitol. The continuous lines represent pore diameters of 4, 6 and 8 Å calculated by means of Eq. (5).

The continuous lines in Fig. 4 represent theoretical A_{sf}/A_{wf} values calculated for cylindrical pores with a diameter d of 4, 6 and 8 Å using Eq. (3.10) of House (1974) for solutes of increasing molecular diameters:

$$A_{sf}/A_{wf} = [2(1-\alpha)^2 - (1-\alpha)^4] \cdot [1 - 2.104\alpha + 2.09\alpha^3 - 0.95\alpha^5]/[2(1-\alpha')^2 - (1-\alpha')^4] \cdot [1 - 2.104\alpha' + 2.09\alpha'^3 - 0.95\alpha'^5] \quad (5)$$

It may be seen that the experimental results can be fitted with a pore diameter between 4.3 and 4.7 Å.

Discussion

The main conclusion from the present work is that (of the probing molecules tested here) the water channel is permeated only by formamide. Formamide has high olive oil/water (75 × 10^{-5}) and hexadecane/water (42 × 10^{-6}) partition coefficients (Whittembury et al., 1993). This indicates that the high P_s for formamide of 84 μm/sec may be due in part to formamide permeation through the lipid bilayer. Equation (3) allows to correct A_{sf}/A_{wf} for this flow of formamide by calculating $P_s \cdot V_s/P_{os} \cdot V_w$ (where P_s = 84 ± 4 μm/sec; V_s = 39.8; V_w = 18; P_{os} ranged from 850 to 1,200 μm/sec in the formamide experiments). The correction varied between 0.15 and 0.20 in different experiments. It brings A_{sf}/A_{wf} from 1.00 − 0.62 = 0.38 (which would hold if P_s = 0 for formamide)

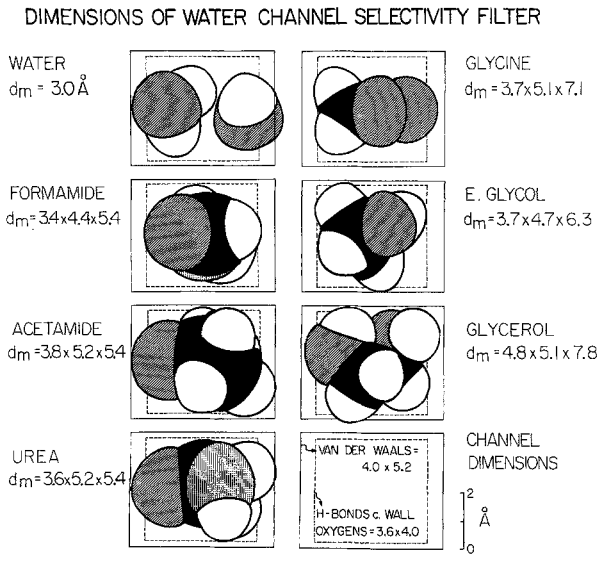

Fig. 5. Silhouettes of water and some of the other molecules used in the present work showing their two smaller diameters (the main diameter would be perpendicular to the plane of the figure) compared with a rectangular possible cross-section of the water channel selectivity filter. The unbroken line is 4.0 × 5.2 Å. The dashed line is 3.6 × 4.0 Å. *See* Discussion.

to 0.16 ± 0.05. Acetamide has also high partition coefficients (olive oil/water, 85 × 10^{-5}; and hexadecane/water, 21 × 10^{-6}). P_s = 1 μm/sec for acetamide, probably because acetamide is a larger molecule than formamide. P_s = 0 for the other probes, and = 0.02 μm/sec for glycerol. The correction of A_{sf}/A_{wf} for these probes is negligible.

As illustrated in Fig. 4, data points for the molecular probes used can be best fit with a pore diameter for the water channel selectivity filter between 4.2 and 4.7 Å. Although the theory used in this treatment refers to ideal cylindrical pores, the spaces in a uniform suspension of fibers do behave as if they were cylindrical (Ogston, 1958). Therefore, our conclusion that the pore diameter lies between 4.2 and 4.7 Å does not necessarily imply that the water channel is a perfect cylinder.

Hille (1992) has pointed out that if the oxygens facing the channel lumen form H bonds with the molecules permeating the channel (in this case water and formamide), the channel "sees" the permeating molecule "smaller" in 0.9 Å than its van der Waals dimensions. If that were the case, the water channel diameter could range from 3.3 to 3.8 Å. But at present we do not have ways of sorting out between these possibilities.

An alternative approximation to the dimensions of the selectivity filter is in Fig. 5. Van der Waal dimension models of the molecular probes are built and compared with a rectangular window through which the probes would pass using their smaller molecular diameters (the longest dimension in Fig. 4 is perpendicular to the plane of the figure). This naive approach sets the channel dimensions as a window of 3.6 × 4.2 Å that would let only water and formamide through, holding back all the other molecules. Two water molecules could not fit on one side of the other within a channel of these dimensions. Thus, there would be single filing. Again, if the channel oxygens form H bonds with water and formamide as they move within the channel, the dimensions of the selectivity filter window would be closer to 3.6 × 4.0 Å. These dimensions compare with the Gramicidin channel (diameter close to 4 Å), through which water single files. This channel also excludes urea, lets formamide through but is permeable to some ions (Cohen, 1975; Andersen & Procopio, 1980; Finkelstein, 1987).

The selectivity filter characteristics of the PST basolateral cell membrane water channel resemble those of the human red cell water channel in that both exclude urea (Macey & Farmer, 1970; Macey, 1984). However, Solomon (1993) has produced evidence that the human red cell water channel does not completely exclude urea.

The observation that σ is one for NaCl is at variance with results of Welling, Welling and Ochs (1987). They obtained a reflection coefficient for NaCl of 0.5. We find that they might have used the wrong equation in their calculations. Also, their experimental conditions during the setup of osmotic steps allow for some volume regulation and might have led them to the wrong conclusion.

It is known that there are urea concentration differences across the tubule wall of the proximal tubule. The observation that σ = 1.00 for urea indicates that urea may be a strong driving force to move water across one single cell membrane. However, if fast urea transporters were present in the cell membrane, they might allow for equilibration of urea on both sides of the membrane on a long-time basis making this possibility unlikely.

Recently, proteins that form water channels in red cells and in the proximal tubule have been identified, cloned and reconstituted into lipid bilayers (Preston et al., 1992; van Hoeck & Verkman, 1992; Zeidel et al., 1992; Agre et al., 1993; Echevarría et al., 1993; Nielsen et al., 1993; Verkman et al., 1993; Zhang et al., 1993). It will be of paramount importance to know whether the expression of these proteins in oocytes and in lipid vesicles do exactly reproduce the channel function as it is in vivo; also, to know whether the selectivity filter of these various water channels are exactly alike.

The help of Mr. J. Mora and of Mrs. D. Otero is highly appreciated. This work was supported in part from grants from CONICIT, Consejo de Desarrollo Científico y Humanístico of UCV and Fundación Polar.

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