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ARTICLE

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Effect of dDAVP on basolateral cell surface water permeability in the outer medullary collecting duct

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Abstract We report a novel approach for assessing the volume of living cells which allows quantitative, high-resolution characterization of dynamic changes in cell volume while retaining the cell functionality. The aim of this study was to evaluate the short-term effect of vasopressin on basolateral cell surface water permeability in the outer medullary collecting duct (OMCD). The permeability of the basolateral cell membrane was determined in the tubules where the apical membrane was blocked with oil injected into the lumen. The apparent coefficient of water permeability (P_f) was evaluated by measuring the cell swelling after the step from hypertonic to isotonic medium (600 mosm to 300 mosm). Desmopressin (dDAVP) induced an increase of the basolateral P_f from 113.7 ± 8.5 μm/s in control cells to 186.6 ± 11.4 μm/s in micro-dissected fragments of the OMCD incubated in vitro (10^{-7} M dDAVP, 30 min at 37 °C) ($P < 0.05$). Mercury caused pronounced inhibition of basolateral water permeability (26.0 ± 6.9 μm/s; $P < 0.05$). The effect of mercury (1.0 mM $HgCl_2$) was reversible: after washing the fragments with PBS for 20 min, P_f values were restored to the control levels (125.0 ± 9.5 μm/s). The results of the study indicate the existence of a mechanism controlling the osmotic water permeability of the basolateral cell membrane in the OMCD epithelium.

Keywords Image analysis · Outer medullary collecting duct · Vasopressin · Water transport

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Introduction

Water movement across cell membranes in the principal cells of kidney collecting ducts occurs during vasopressin signal transduction. In kidney medulla, water transport is driven primarily by osmotic forces created by ion and urea movement, and to a lesser extent by oncotic and hydrostatic forces. At least two water transporting proteins have been localized in the basolateral membrane: AQP3 and AQP4 (Agre 2000; Verkman et al. 2001; Nielsen et al. 2001). These water channels are water-selective integral membrane proteins that function as conduits for osmotically driven transport of water across cell plasma membranes (Meinild et al. 1998; Sasaki et al. 1998; Verkman and Mitra 2000). It has been shown that the water deprivation-induced increase in the water permeability of collecting duct epithelia was due to an increase in AQP2 expression (Terris et al. 1996; Murillo-Carretero et al. 1999). Because AVP levels were increased by water deprivation, it was postulated that AVP mediates this increase. In vivo and in vitro studies have demonstrated that regulation of collecting duct epithelia cell water permeability by AVP occurs by reversible translocation of AQP2 from intracellular vesicles to the apical plasma membrane (the shuttle hypothesis) (Nielsen et al. 1995). Two other water channels, AQP3 (Ecelbarger et al. 1995) and AQP4 (Terris et al. 1995), are also located in the principal cells of the collecting duct epithelia. It was believed that both are expressed in the basolateral membrane constitutively and form the exit pathway for water. Nevertheless, Northern blot and immunoblot analysis showed that expression of AQP3 were increased in response to water deprivation. The physiological significance of the increased levels of mRNA and protein of these water channels in collecting duct epithelia remains to be established. The quantitative measurement of water permeability could be a useful tool in studies of aquaporin function and regulation.

One of the main parameters in the characterization of osmotic water permeability is the osmotic water

permeability coefficient, P_f (Verkman et al. 1996). There are two main approaches to measure water movement across cell membranes: observation of the volume of the membrane vesicles or of the whole cell during osmotic shock (Flamion et al. 1995; Verkman 1995; Farinas and Verkman 1996; Maric et al. 2001), and tracing the changes of marker compound in the cytoplasm or inside membrane vesicles (Martial and Ripoche 1991; Farinas et al. 1995; Zelenina and Brismar 2000). However, these approaches present technical difficulties and there are some illustrations of the problems involved. Techniques utilized for cell volume measurements so far include: (1) measurement of the intensity of the scattered light produced by either cells or membrane vesicles; (2) ultrarapid filtration methods to measure the content of tritiated water in membrane vesicles; (3) concentration changes of cellular markers measured by fluorescent emission; and (4) light microscopic observation by cinematography of cell outlines at a fixed depth (Rivers et al. 1996; Solenov et al. 2001). The measurement of osmotic water permeability requires volume measurement on a fast timescale. All these methods are suitable to follow fast volume changes caused by osmotic challenge.

The present paper describes a new approach to measure water permeability of the basolateral surface of the kidney collecting ducts. Short fragments of the outer medullary collecting duct (OMCD) were injected with mineral oil to block apical water flow. Digital movies of the swelling epithelia were recorded during osmotic shock. For analysis of the images we model the cell volume change as a function of time and selected area of the epithelia image obtained from the experiment. The equations obtained were used to yield the best fit to the experimental data and to find P_f values. We apply this procedure to determine the effect of water deprivation and the vasopressin agonist dDAVP on water permeability of the basolateral surface of the kidney collecting ducts.

Materials and methods

Methods

Animals

Wistar rats weighting 150–200 g (Breeding Laboratory of Experimental Animals, Institute of Cytology and Genetics, Novosibirsk) were kept in individual cages and received 5% sucrose in water during 48 h; other food was restricted. Before the experiments the rats were anaesthetized with pentobarbital (50 mg/kg i.p.), the kidneys were extracted and placed in ice-cold PBS, and a suspension of collecting duct fragments was prepared.

Perfusion chamber and microscopy

A perfusion chamber was constructed as an acrylic block mounted on the objective of the microscope (water immersion ×65 magnification, numerical aperture 1.1, 1.6×magnification photo adapter). The perfusate flow rate was generally 30 mL/min, which gave complete perfusate exchange in under 100 ms. The glass plate bearing the fragments of the OMCD was positioned on the stage of a microscope (MIKMED-2, LOMO, St. Petersburg, Russia) for acquisition of transmitted light images by a CCD camera (Webcam 3, Creative Technology). Twelve-bit monochrome images were captured at a rate of 15 frames per second, stored on a personal computer, and archived on CD disks. Software for image acquisition was supplied with the camera (Videoblaster WebCam Control 2.14, Creative Technology). Images were processed by Adobe Photoshop 3.0 (Adobe Systems). Figure 1 shows our experimental setup and approach to image analysis.

Collecting duct fragments

Tubules from the OMCD were dissected from pathogen-free Wistar rats. The kidneys were isolated and tissue from the outer medulla zone was cut from the kidneys and placed in a syringe containing ice-cold calcium-free PBS. Then the tissue was squeezed through a needle (0.45 mm i.d.) in the same ice-cold calcium-free PBS. The resulting suspension was filtered through nylon mesh, diluted 10 times with MEM culture medium and centrifuged (100×*g*, 10 min, 4 °C). Sediment containing the

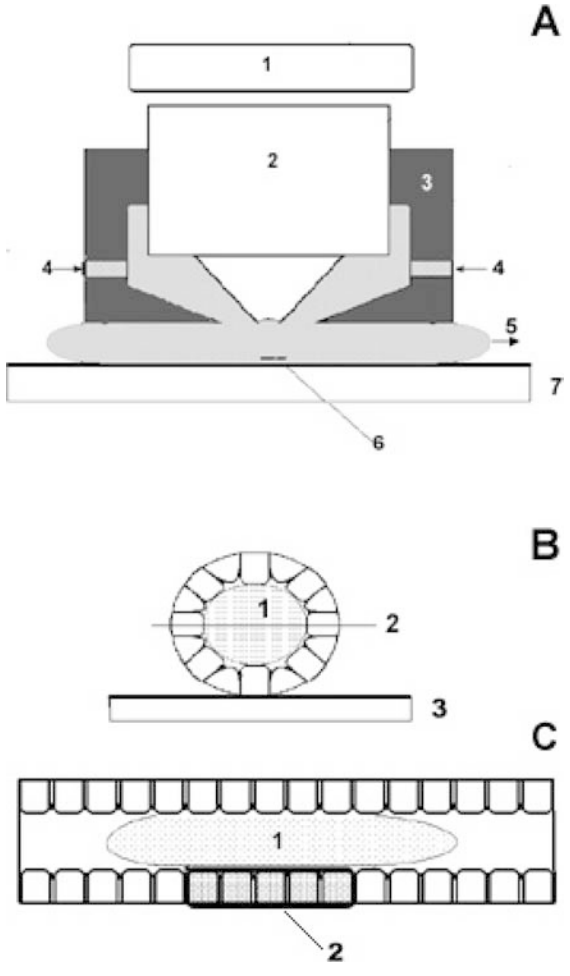

Fig. 1 **A** Perfusion experimental setup: *1*, CCD camera; *2*, objective lens; *3*, acrylic block; *4*, perfusate; *5*, suction; *6*, collecting duct fragment; *7*, glass plate. **B** Schematic diagram of the tubule fragment: *1*, oil droplet; *2*, focal plane; *3*, glass plate. **C** Cross section of tubule: *1*, oil droplet; *2*, selected area of interest

tubules was diluted with MEM culture medium to an appropriate concentration of about 5–10 fragments per microliter. This suspension was used in experiments as a preparation of OMCD fragments. To immobilize the fragments, 10 or 20 μL of suspension were transferred into a drop of medium on the glass plate covered with poly-D-lysine (Sigma, St. Louis, USA), and the suspension was left for sedimentation. An OMCD fragment was chosen according to morphological criteria, and an oil droplet was injected into the lumen using a glass micropipette. The fragment was then placed under the perfusion chamber on the microscope stage.

Water permeability measurements

The solutions used were based on PBS and contained 1.0 mg/mL glucose in which mannitol was added to increase the osmolarity. This solution was chosen so as to be able to degas it without affecting its pH. To create osmotic challenges, perfusate solutions were changed from hypertonic PBS to normal PBS. Fragments of the OMCD were scanned with a water immersion objective (Fig. 2A). The hypertonic (H) bathing solution (600 mosm/kg; 300 mosm PBS with 300 mM mannitol, ICN Biomedicals) was applied. Cell swelling was then initiated by changing the bathing solution to normal (N) PBS (300 mosm/kg). The image recording was started 1–2 s before the change from H to N bathing solution and continued for 10 s. The osmotic challenges were performed and cell swelling was monitored on the microscope at 37 °C. Fragments were tested according two protocols: long and short. The short protocol was:

1. Measurement of the swelling kinetics of the intact epithelium (intact control).
2. Incubation with 10^{-7} M desmopressin (dDAVP) (Sigma, USA) during 30 min at 37 °C.
3. Measurement of the swelling kinetics.

The long protocol was the three steps of the short protocol plus four additional steps:

4. Incubation with 1.0 mM $HgCl_2$ during 20 min at room temperature.
5. Measurement of the swelling kinetics.
6. Washing with PBS (20 min, flow rate 0.5 mL/min, room temperature).
7. Measurement of the swelling kinetics (second control).

Data analysis

The plasma membrane osmotic water permeability coefficient (P_f) was calculated from the time course of the volume change in response to an osmotic gradient. Osmotic water movement is the net flow of volume across a membrane in response to a hydrostatic and/or osmotic pressure:

$$J = -L_p S_e \Delta\phi(t); \Rightarrow dV/dt = -P_f S_e V_w \Delta C(t) \quad (1)$$

where J is the rate of volume flow across the membrane ($cm^3\ s^{-1}$), (dV/dt) is the rate of cell volume change, L_p is hydraulic conductivity ($cm^3\ s^{-1}\ atm^{-1}$), P_f is the osmotic water permeability coefficient ($cm\ s^{-1}$), S_e is the surface area (cm^2) which is significant for water exchange, $\Delta\phi(t)$ is the osmotic pressure difference (atm) at time t, $\Delta C(t)$ is the osmotic concentration difference (osm kg^{-1}) at time t, and V_w is the partial molar volume of water.

Raw data from experimentally obtained radial section areas (A) during the swelling after the osmotic challenge, assuming that cell shape changes occur only in the radial direction, could be fitted to the exponential function:

$$A(t) = A_{min} + (A_{max} - A_{min})(1 - \exp(-t/\tau)) \quad (2)$$

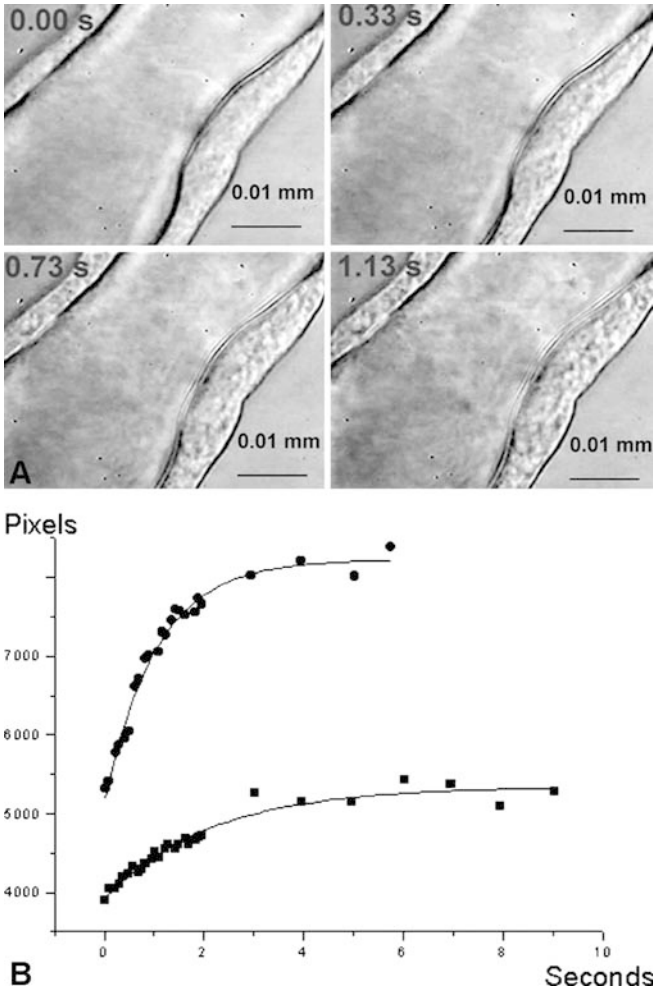

Fig. 2 **A** Swelling process of the epithelia of the outer medullary collecting duct. A collecting duct fragment from the outer medulla injected with oil was initially equilibrated in hypertonic bathing solution. Cell swelling was then initiated by changing the solution to normal PBS; after the osmotic challenge the cells undergo a small but measurable swelling. **B** Time course of the experimentally obtained radial section areas of the epithelia during the swelling after the osmotic challenge. The length of the selected image area (parameter l in Eq. 9) has the same value in the control and actual experiments. *X*-axis: seconds; *Y*-axis: area of selected image part (pixels). *Squares*: control; *circles*: effect of dDAVP

where τ represents the time constant of the swelling process (Maric et al. 2001).

At the beginning of this time course curve the changes of the cell volume could be assumed as linear. The osmotic gradient could be assumed unchanged during this period:

$$\Delta C = (C_{in} - C_{out}) = \text{const.} \quad (3)$$

where C_{in} is the osmotic concentration inside the cell and C_{out} is the osmolality of the bathing solution.

According to this assumption, experimental data were fitted to the linear function and we could use the simplified formula of Fick's law to calculate P_f:

$$(V_1 - V_0)/(t_1 - t_0) = P_f V_w S_e (C_{in} - C_{out}) \quad (4)$$

$$P_f = (V_1 - V_0)[(t_1 - t_0) V_w S_e (C_{in} - C_{out})]^{-1} \quad (5)$$

The unit surface area across which water is exchanged and the epithelium volume could be calculated from a cylinder surface formula, where the angle is equal to 1:

$$S_e = Rl \tag{6}$$

$$V = hRl \tag{7}$$

where R is the radius of the collecting duct fragment, h is the height of the epithelium, and l is the length of selected image area.

The radial section area obtained from the image is $A = hl$. Substituting V by its value defined in Eq. 7 leads to:

$$P_f = (A_1 - A_2)[(t_1 - t_0)V_w l(C_{in} - C_{out})]^{-1} \tag{8}$$

The experimentally obtained data $(A_1-A_2)/(t_1-t_0)$ can be changed for the coefficient of linear regression (K_r). The permeability coefficient of the basolateral surface of the epithelium can be calculated from the slope (K_r) of the linear plot:

$$P_f = K_r[V_w l(C_{in} - C_{out})]^{-1} \tag{9}$$

Results

The approach used to measure the osmotic water permeability coefficient based on cell swelling results in an increase in cell height, whereas lateral movement is not evident (Maric et al. 2001), as indicated by the insignificant changes of the fragment length during the swelling process in our experiments (data not shown). This leads to the assumption that the radial section area of each cell might serve as an appropriate parameter to monitor cell volume changes in response to osmotic challenges. Figure 2 shows the typical time course curves of epithelia swelling. The time courses of cell swelling for OMCD fragments were established under four types of conditions: swelling under control conditions, swelling after treating with dDAVP, swelling after treating with $HgCl_2$ and swelling after removing of mercury. The effect of desmopressin on basolateral water permeability was studied on OMCD fragments taken from water-loaded rats. Endogenous vasopressin is significantly inhibited in these animals. The low level of vasopressin leads to a low expression of vasopressin-dependent water channels in the cell plasma membrane. The effect of desmopressin is more pronounced in water-loaded animals. The effect of mercury was studied on fragments treated with desmopressin for which the water permeability was enhanced. The total time for testing a fragment in the long protocol (see Methods) did not exceed 80 min. The measurements were done during the initial period of swelling when change of the cell volume could be assumed as a linear function. As described in Methods, the coefficient of linear regression of the cell volume or the experimentally obtained radial section area could be used as a measure of the rate of osmotic cell swelling:

$$P_f = K_r[V_w l(C_{in} - C_{out})]^{-1} \tag{10}$$

A glass plate with OMCD fragments staked on poly-D-lysine were placed on the stage of the microscope and an oil droplet was injected into the lumen using a glass micropipette. The perfusion chamber was placed over the chosen fragment and the H solution was applied with an equilibration time of 15 s; then the H solution was changed to N to cause the swelling process (Fig. 2A). This swelling experiment was repeated twice. The P_f values obtained in these tests were used as control values for subsequent tests of this fragment. Then the fragment was incubated in the presence of 10^{-7} M dDAVP and the swelling tests were repeated in duplicate. The plotted values of the radial section areas represent the time course of the cell swelling (Fig. 2B).

The water permeability (P_f) was calculated using the quasi-linear slope of the initial part of the time course of the swelling process. In our experiments the quasi-linear part lasted about 0.7 s; for calculation, this part of the curve (0.7 s, 10 frames) was used. The whole curve could be fitted by an exponential decrease function. The increase of the epithelial volume was less than twice in these experiments, probably because of limited osmotically active cell volume and/or activation of the regulatory volume decrease mechanism. The significant increase of P_f compared with the control was revealed for the fragments treated with 10^{-7} M dDAVP (mean ± SE: 113.7 ± 8.5 μm/s, $N = 11$, and 186.6 ± 11.4 μm/s, $N = 11$) and a pronounced inhibitory effect of mercury on basolateral water permeability was shown (26.0 ± 6.9 μm/s, $N = 7$). The effect of mercury was reversible. After washing the fragments with PBS for 20 min, the P_f values were restored to the control levels (125.0 ± 9.5 μm/s) (Fig. 3).

The control levels for water permeability obtained in these experiments are in good agreement with data from

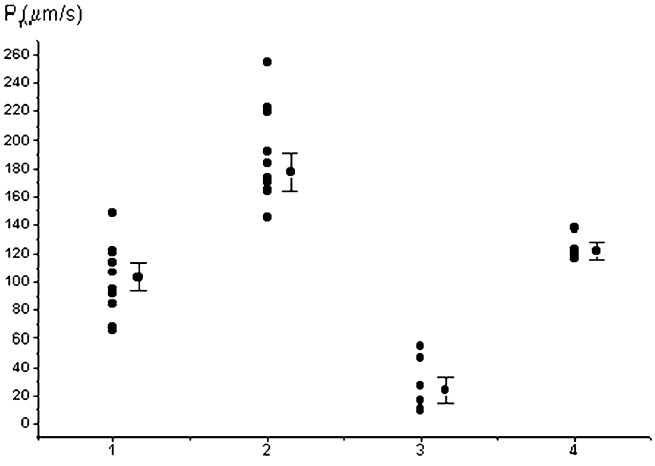

Fig. 3 Effect of desmopressin and mercury on the osmotic water permeability of the basolateral surface of the OMCD. P_f was measured: *1*, before treating with dDAVP (first control); *2*, after incubation with desmopressin (10^{-7} M dDAVP, 30 min, 37 °C); *3*, then after the same fragment was treated with mercury (1.0 mM $HgCl_2$, 20 min, room temperature); *4*, after removing mercury with PBS (second control)

Flamion and Spring (1990). The inhibitory effect of mercury indicates a significant contribution of mercury-sensitive water channels in basolateral osmotic water permeability. These findings correspond well to the data about AQP3 expression in the OMCD (Ecelbarger et al. 1995).

Discussion

The goal of this study was to develop a simple, quantitative method to measure plasma membrane water permeability in epithelial cells which does not require expensive hardware equipment. The motivation for this study was the limitation of existing methods and the need to make permeability measurements on intact tissues. The instrumentation required to carry out the permeability measurements includes only a conventional phase contrast microscope, a micro-manipulator, a CCD camera and a cell perfusion chamber. The perfusion chamber is an important component of the experimental setup. The chamber should permit solution exchange with rapid exchange times and little turbulence to minimize the movement of an isolated kidney tubule during the process of image recording. A chamber was constructed for rapid fluid exchange and minimal turbulence. The method described for measurement of the osmotic water permeability of the basolateral surface of OMCD fragments permits the quantitative real-time monitoring of changes in thickness of the epithelial cells.

This method can be placed between one-dimensional methods like the fluorescent microbeads method or scanning ion conductance microscopy and three-dimensional methods with preloaded fluorophores. A two-dimensional parameter appears to be adequate because in our experiments the volume changes of the cells in the epithelial layer are tracked by only one degree of freedom during the process of swelling or shrinkage, namely that of cell height. There is negligible lateral membrane displacement (Farinas and Verkman 1996; Maric et al. 2001). Data reported by different authors comparing one- and three-dimensional approaches to measure osmotically induced cell volume kinetics (Crowe and Wills 1991; Van Driesshe et al. 1993; Korchev et al. 2000), as well as our results, indicate the validity of approaches omitting the calculation of the total cell volume. This method leads to valid results without the requirement for pretreatment of cells with volume indicators and does not require calibration to obtain quantitative data. The changing period from solution ''H'' to solution ''N'' was less than 100 ms and the swelling rate was measured during the initial period of the process when the growing cell height was 10–15%.

It is important to distinguish between mechanisms that determine the steady-state volume and mechanisms that correct acute volume perturbation. Not distinguishing between these mechanisms can be a source of confusion and it is best to consider these as separate processes. Transporters and processes responsible for correcting acute changes in cell volume are usually not active at the steady state. Thus the steady-state cell volume is not a balance between ongoing, opposite actions. Cells appear to activate volume-regulatory transporters only when the cell volume deviates from a ''set point''. Because of the small changes in cell volume in our experiments, the RVD process was negligible. During the initial period the cell volume kinetics are assumed to be linear and the swelling process could be described by Fick's law.

Within several seconds the rate of cell volume change deviates from that predicted by an ideal osmometer. The mechanism accounting for this deviation is unclear. The reason for inhibition of the volume increase during cell swelling could be a deficiency of the osmotically active volume, and/or activation of leakage of cell osmolites, which is a significant component of the RVD process. Aquaporins AQP3 and AQP4 have been shown to provide the principal route for osmotically driven water transport across the basolateral membranes of collecting duct principal cells (Agre 2000; Nielsen et al. 2001; Verkman et al. 2001). Our method was applied to determine the role of the V_2 vasopressin receptor in regulation of basolateral water permeability of OMCD epithelial cells, where AQP3 constitutes the main pool of water channels of OMCD cells' basolateral membranes. It has been shown that vasopressin enhances the expression of this aquaporin (Ecelbarger et.al. 1995). The published data indicate that AQP2 probably contributes to the water permeability of the basolateral plasma membrane. AQP2 has been shown by immunostaining in the basolateral area in inner medullary collecting duct principal cells (Nielsen et al. 2001). AQP2 immunostaining of low intensity has been found to be associated with the principal cell basolateral plasma membrane in the OMCD. The role that AQP2 plays in the OMCD basolateral water permeability remains to be established.

The results of our study show that dDAVP, an agonist of the V2 receptor, caused a significant increase of osmotic water permeability of the basolateral surface of OMCD fragments. We can suppose that V2 receptors play a significant role in the regulation of basolateral water permeability in the CD. High concentrations of oxytocin can increase basolateral water permeability in CDs through the V2 receptor (Jeon et. al. 2003). The inhibition of osmotic water permeability by mercury, which was shown in our experiments, is in good agreement with data which indicate that the pool of water channels in the basolateral membranes of OMCD cells mainly consists of AQP3.

Considered together, the results presented here and the published data suggest that enhanced expression of aquaporins AQP2 and AQP3 probably contributes to increase the osmotic water permeability of the basolateral surface of the OMCD.

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