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Asymmetry in the Osmotic Response of a Rat Cortical Collecting Duct Cell Line: Role of Aquaporin-2

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Abstract. Transition from antidiuresis to diuresis exposes cortical collecting duct cells (CCD) to asymmetrical changes in environment osmolality, inducing an osmotic stress, which activates numerous membrane-associated events. The aim of the present work was to investigate, either in the presence or not of AQP2, the transepithelial osmotic water permeability (P_{osm}) following cell exposure to asymmetrical hyper- or hypotonic gradients. For this purpose, transepithelial net volume fluxes were recorded every minute in two CCD cell lines: one not expressing AQPs (WT-RCCD$_1$) and another stably transfected with AQP2 (AQP2-RCCD$_1$). Our results demonstrated that the rate of osmosis produced by a given hypotonic shock depends on the gradient direction (osmotic rectification) only in the presence of apical AQP2. In contrast, hypertonic shocks elicit P_{osm} rectification independently of AQP2 expression, and this phenomenon may be linked to modulation of basolateral membrane permeability. No asymmetry in transepithelial resistance was observed under hypo- or hypertonicity, indicating that rectification cannot be attributed to a shunt through the tight junction path. We conclude that osmotic rectification may be explained in terms of dynamical changes in membrane permeability probably due to activation/incorporation of AQPs or transporters to the plasma membrane via some mechanism triggered by osmolality.

Key words: Renal cell line — AQP2 — Transepithelial volume fluxes — Hydrostatic and osmotic permeability — Cell volume regulation

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Introduction

Water reabsorption in the mammalian cortical collecting duct (CCD) is important for the overall operation of the urinary concentrating mechanism. During this process, CCD cells need to monitor prevailing luminal and interstitial osmolalities to modulate vectorial fluid transport. In physiological conditions acute increases in plasma osmolality (anti-diuretic state) leads to liberation of arginine-vasopressin (AVP) and CCD cells become water-permeable as a result of the apical insertion of aquaporin-2 (AQP2). Water is then passively reabsorbed into the near isotonic interstitium, leading to increases in luminal osmolality [9, 29]. On the other hand, during the diuretic state, caused by inhibition of AVP release, AQP2 resides in intracellular vesicles of principal cells of the CCD. This location prevents water reabsorption and excessive amount of a hypotonic fluid enters the medulla [1].

Therefore, during transition from antidiuresis to diuresis, cells of the CCD are faced with asymmetrical changes in external osmolality, which would modify both transepithelial fluid transport (J_v) and osmotic permeability (P_{osm}).

Several studies in isolated perfused collecting tubules have reported asymmetry in the osmotic response, indicating that the rate of osmosis produced by a given gradient depends on the direction of the gradient (osmotic rectification) [37, 38]. In the rabbit CCD, Schafer et al. (1974) provided evidence of rectification in the ADH-independent, but not in the ADH-dependent, osmotic flow [36, 37]. On the other hand, Kuwahara et al. (1991) proposed that vasopressin-independent and -dependent water transports are symmetrical and not altered for at least 10 min following a change in bath osmolality [24]. Some authors proposed that asymmetry in osmotic flow could be the result of flow-dependent changes in

the dissipative permeability characteristics of the tight junctions [37], while others suggested that it could be due to changes in the osmotic permeability of the rate-limiting barrier as a consequence of changes in cell volume and/or cell morphology [23, 38, 41]. Thus, osmotic flow rectification in cortical collecting tubule is not completely understood.

Fischbarg (1997) reported that fluid transport can be explained in terms of asymmetrically distributed regulatory volume mechanisms and is dependent on cyclic cell volume changes [15]. We have previously demonstrated, in a rat collecting duct cell line, $RCCD_1$, which exhibits many major functional properties of CCD [2, 11, 12], that exposition to hyper- or hypotonic gradients induced cell shrinkage or swelling, but activation of regulatory volume increase or decrease mechanisms (RVI or RVD) was dependent on the locus of the osmotic medium, i.e., whether present on the apical or basolateral surface [17, 18]. The differential activation of regulatory volume mechanisms raises the question of whether asymmetrical osmotic gradients may also conduce different transepithelial osmotic water fluxes. Therefore, the aim of the present work was to characterize the water permeability, either in the presence or not of AQP2, following cell exposure to asymmetrical hyper- or hypotonic gradients. For this purpose we have used two cortical collecting duct cell lines: one not expressing AQPs (WT-$RCCD_1$) [7] and another stably transfected with AQP2 (AQP2-$RCCD_1$) [17]. Our results show that hypotonic shocks only induced P_{osm} rectification in the presence of AQP2 and this response is dependent on changes in the apical membrane permeability. On the other hand, hypertonic shocks elicit P_{osm} rectification, independently of AQP2 expression, and this phenomenon is linked to modulation of basolateral membrane permeability. We conclude that osmotic rectification may be explained in terms of dynamical changes in membrane permeability probably due to activation/incorporation of AQPs or transporters to the plasma membrane via some mechanism triggered by osmolality.

Materials and Methods

Cell Culture

$RCCD_1$ cells were grown in DM medium (Dulbecco's modified Eagle's medium / Ham's F-12, 1:1 v/v; 14 mM $NaHCO_3$, 3.2 mM glutamine; 5 μg/ml transferrine; 5×10^{-8} M dexamethasone; 3×10^{-8} M sodium selenite; 5 μg/ml insulin; 10 μg/ml epidermal growth factor; 5×10^{-8} M triodothyronine; 10 units/ml penicillin - streptomycin; 20 mM HEPES; pH 7.4) and 2% fetal bovine serum (FBS) (Gibco BRL) [2]. All experiments were performed on confluent cells, between the 20th and 40th passages, grown on permeable filters during six or seven days (Transwell, 3 μm pore size, 4.5 cm^2 growth area, Corning Costar Cambridge, MA).

AQP2-$RCCD_1$ cells, stably transfected with cDNA coding for rat AQP2, were maintained in DM medium containing Geneticin (400 μg/ml, Life Technologies) as previously reported [17].

Transepithelial Net Volume Flux Measurements

Confluent $RCCD_1$ cells grown on filters were directly inserted between two barrel-shaped lucite hemi-chambers. One of them (basolateral) was open to the atmosphere, while the other (apical) was hermetically sealed. The closed chamber was connected with a small-diameter polyethylene tube to the net water measurement system where the net volume flux (J_v) was recorded every minute, as previously reported [13]. Briefly, the position of a liquid meniscus inside a capillary tube was photoelectrically detected. Displacements to the right or to the left were proportional to the amount of water moving across the monolayer. The sensitivity of the system was 0.05 $\mu l \cdot min^{-1}$. J_v values were normalized per monolayer surface area, then expressed in $\mu l \cdot min^{-1} \cdot cm^{-2}$. The basolateral bath was continuously bubbled with the appropriate CO_2/O_2 mixture to maintain the pH of the medium at 7.4 ± 0.1 (37°C).

J_v measured in the presence of hydrostatic (ΔP) or osmotic (ΔOsm) gradients is described by:

$$J_v = P_{hydr} \cdot \Delta P$$

$$J_v = P_{osm} \cdot \Delta Osm$$

Where P_{hydr} and P_{osm} can be calculated from the slope of the regression line obtained when J_v values are plotted against ΔP or ΔOSM, as previously described [14]. To express P_{hydr} and P_{osm} in $cm \cdot s^{-1}$ the V_w (volume of 1 mol of water), R (gas constant) and T (thermodynamics temperature) were employed.

In some experiments transepithelial resistance (R_T) was measured with a Millicell Electrical Resistance System (ERS, Millipore) after apical or basolateral osmotic shocks.

Solutions and Chemicals

In transepithelial water movement studies, cells were bathed on each side with a "standard saline solution" containing (in mM): 139 NaCl, 5 KCl, 10 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$, 0.8 $MgSO_4$, 20 HEPES, 5 glucose, pH 7.4. In Na^+-free experiments (0 Na^+), NaCl was iso-osmotically replaced by choline chloride. In pharmacological experiments, the inhibitors ouabain (10^{-4} M) or potassium cyanide (KCN, 8.3×10^{-3} M) were assayed (Sigma & Aldrich) and vehicles (ethanol or water), used to prepare drugs, were tested in control experiments.

In order to maintain the ionic strength, anisosmotic solutions were prepared by mannitol addition or removal to the isosmotic solution containing (in mM): 90 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 0.8 $MgSO_4$, 1 $MgCl_2$, 100 mannitol, 20 HEPES, 5 glucose, osmolality: 320 ± 4 mOsm. The osmolalities were routinely measured by a pressure vapor osmometer (Wescor, Logan, UTAH). All solutions were titrated to pH 7.4 using Tris (Sigma & Aldrich) and bubbled with atmospheric air.

Data Analysis and Statistics

In pharmacological studies $RCCD_1$ cells were cultured in six holder clusters. To minimize random fluctuations, 3 holders were routinely taken as control and the other 3 were tested with the employed agents. Results are expressed as mean values ± SE. Student's *t*-test was used for paired or unpaired data and ANOVA with a Scheffe *ad-hoc* test was used for multiple comparisons. A $p < 0.05$ was considered significant.

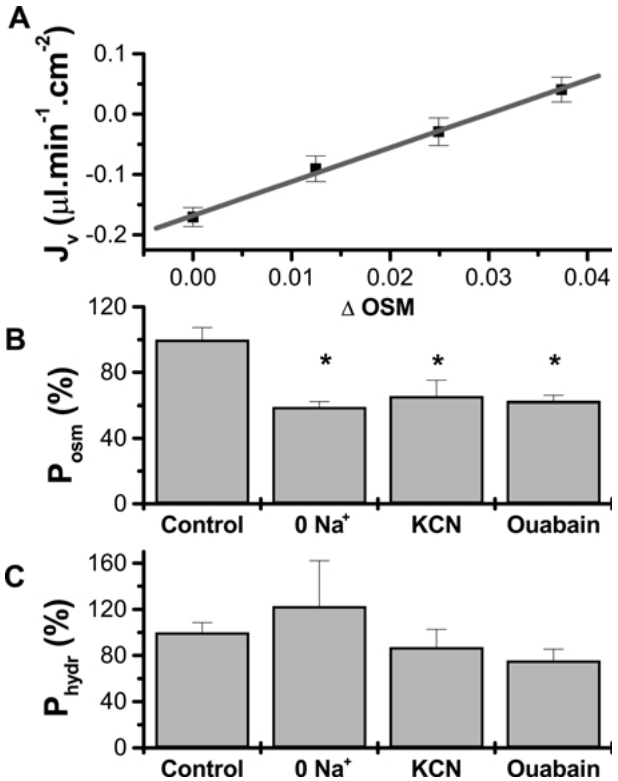

Fig. 1. Water permeability in WT-RCCD$_1$ cells. (*A*) Transepithelial volume fluxes (J_v, µl · min^{-1} cm^{-2}) measured as a function of basolateral hypertonic gradients (ΔOsm), generated by mannitol addition to the bath, in confluent WT-RCCD$_1$ cells grown on filters. The osmotic permeability coefficient (P_{osm}) was calculated from the slope of the curve as described under "Material and Methods", giving a value of $(1.520 \pm 0.053) \times 10^{-3}$ cm.s^{-1}, $n = 12$. (*B*) P_{osm} was evaluated after Na^+ removal ($n = 7$) and after KCN (8×10^{-3} M, $n = 6$) or ouabain (10^{-4} M, $n = 5$) addition to the bath. Data are expressed as percentage of control values; $^*p < 0.05$, ANOVA with a Scheffe *ad-hoc* test for multiple comparisons. (*C*) Effects of Na^+ removal ($n = 3$) and KCN ($n = 4$) or ouabain ($n = 4$) addition on hydrostatic permeability coefficients (P_{hydr}) expressed as percentage of control values ($n = 6$). Values are not significantly different with respect to control condition (ANOVA with a Scheffe *ad-hoc* test for multiple comparisons).

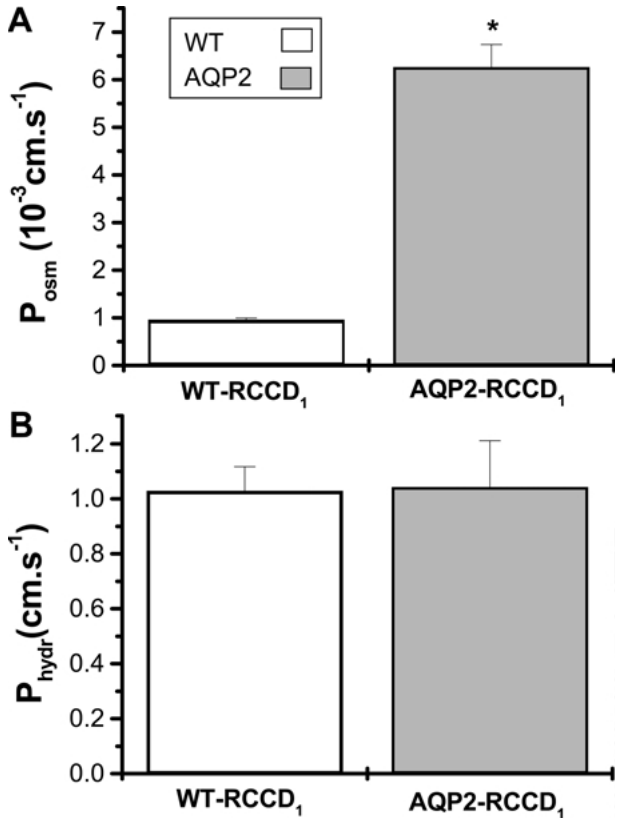

Fig. 2. Water permeability in AQP2-RCCDi cells. (*A*) Comparison between P_{osm} values obtained by increasing basolateral osmolality with mannitol (up to 400 mOsm), in WT-RCCD$_1$ and AQP2-RCCD$_1$ cells; $^*p < 0.0001$, $n = 8$, Student's *t*-test for unpaired data. (*B*) Comparison between P_{hydr} values obtained after cell exposure to hydrostatic pressure gradients (ΔP), in WT-RCCD$_1$ cells and in AQP2-RCCD$_1$ cells.

Results

Osmotic and Hydrostatic Water Permeability in WT-RCCD$_1$ Cells

Transepithelial volume fluxes (J_v) were continuously measured in WT-RCCD$_1$ cells after basolateral increases in osmolality (ΔOSM). Fig. 1*A* shows a linear correlation between J_v and ΔOSM ($r = 0.99, n = 5$). The osmotic permeability coefficient (P_{osm}) was obtained from the slope of the regression line as described under Material and Methods. It can be observed that P_{osm} values were significantly reduced either after Na^+ removal from the bath or in the presence of metabolic inhibitors like potassium cyanide (KCN) or ouabain (Fig. 1*B*). These results suggest that in WT-RCCD$_1$ cells, at least, a fraction of the observed osmotic flux (J_v^{osm}) is dependent on Na^+ transport and is associated with cellular metabolism.

Cell exposure to different hydrostatic pressures (ΔP) also resulted in a linear relationship between J_v and ΔP ($r = 0.95$, $n = 5$). However, the hydrostatic permeability coefficient (P_{hydr}) was not significantly different either in the absence of Na^+ or in the presence of KCN or ouabain (Fig. 1*C*), suggesting that P_{hydr} is independent of active cellular ions transport.

Osmotic and Hydrostatic Water Permeability in AQP2-RCCD$_1$ Cells

We have previously demonstrated that AQP2-RCCD$_1$ cells constitutively express AQP2 in the apical membrane [17]. Now, transepithelial volume flux experiments showed that P_{osm} was significantly increased after AQP2 transfection (Fig. 2*A*). P_{osm} was not affected by ouabain in AQP2-RCCD$_1$ cells

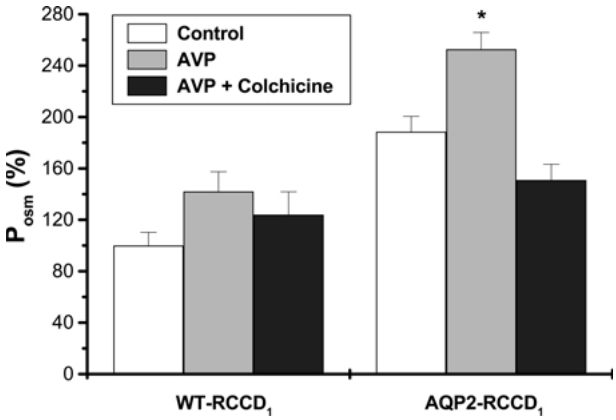

Fig. 3. AVP regulation of P_{osm} in WT-RCCD$_1$ and AQP2-RCCD$_1$ cells. P_{osm} values were obtained in confluent cells grown on filters incubated either without hormone (*control*), or in the presence of 10^{-8} M AVP, or both 10^{-8} M AVP and 10^{-4} M colchicine added to the basolateral medium. Experimental values are mean ± SE, expressed as percentage of WT-RCCD$_1$ cells' control values. $^{*}p < 0.01$, Student's *t*-test for unpaired data ($n = 9$).

(% of inhibition: 5 ± 8, $n = 13$, N.S). On the other hand, P_{hydr} was not significantly different between WT-RCCD$_1$ and AQP2-RCCD$_1$ cells, probably indicating a paracellular fluid movement not affected after AQP2 expression (Fig. 2*B*).

AQP2-RCCD$_1$ Cells Are Able to Modulate the Osmotic Water Permeability

In WT-RCCD$_1$ and AQP2-RCCD$_1$ cells, P_{osm} values, obtained after increasing basolateral osmolality, were evaluated following 10^{-8} M AVP and/or 10^{-4} M colchicine addition to the basolateral bath (Fig. 3). In WT-RCCD$_1$ cells P_{osm} was not significantly modified in the presence of AVP or AVP + colchicine. These results confirm that the hydro-osmotic response is completely lost in the absence of AQPs. In contrast, in AQP2-RCCD$_1$ cells an additional P_{osm} increment was observed after AVP stimulation and this response was abolished after cell exposure to colchicine. These results strongly suggest that AQP2-RCCD$_1$ cells retain the capacity of incorporating functional AQP2 from intracellular vesicles to the plasma membrane.

Asymmetry in the Osmotic Response of WT-RCCD$_1$ and AQP2-RCCD$_1$ Cells

The next series of experiments was performed to further investigate the characteristics of the osmotic response, in both cell lines, after asymmetrical changes in luminal or serosal osmolality. Figure 4*A* and *B* summarizes the time course of osmotic volume flows (J_v^{osm}), in WT-RCCD$_1$ and AQP2-RCCD$_1$ cells, after exposure to hypotonic (−100 mOsm mannitol) or hypertonic (+100 mOsm mannitol) solutions. In

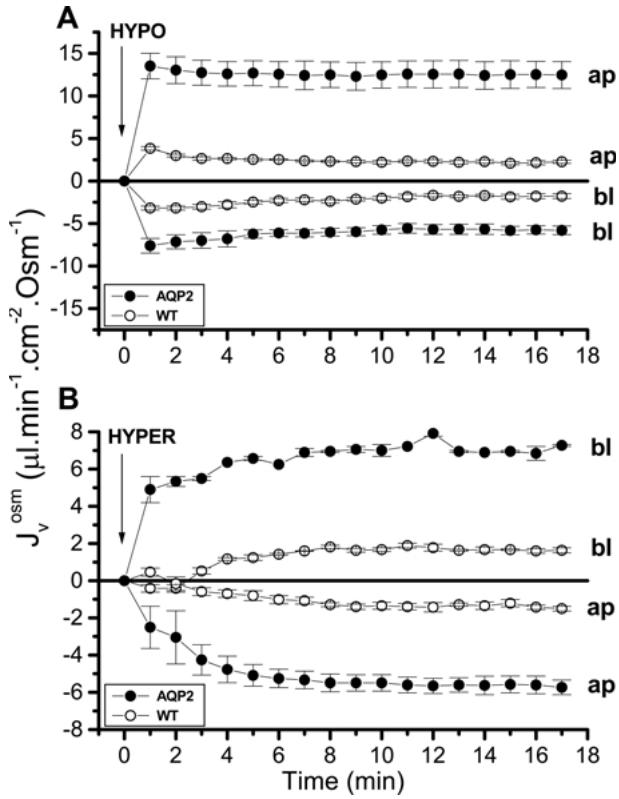

Fig. 4. Time course of osmotic volume flux (J_v^{osm}) in WT-RCCD$_1$ and AQP2-RCCD$_1$ cells. J_v^{osm} were measured in the presence of hypotonic medium, achieved by subtracting 100 mOsm mannitol from the iso-osmotic solution (*A*) or in the presence of hypertonic medium generated by the addition of 100 mOsm mannitol to the iso-osmotic solution (*B*). Cells were exposed to osmotic shocks either from the basolateral (bl) or the apical (ap) bath. Data are mean ± SE of 6 to 8 independent experiments.

all experimental conditions, positive values indicate absorptive fluxes (apical-to-basolateral), while negative values represent secretory ones (basolateral-to-apical). The results show that in AQP2-RCCD$_1$ cells J_v^{osm} (secretory or absorptive) were always significantly higher than in WT-RCCD$_1$ cells. Under hypotonic shocks, asymmetrical osmotic fluxes were only observed in AQP2-RCCD$_1$ cells. On the other hand, asymmetry in the osmotic response was elicited upon hypertonic shocks in both cell lines.

Figure 5 summarizes P_{osm} values, calculated at the steady state (~10–15 min), after hypo- or hypertonic challenges induced either from the basolateral (P_{osm}^{bl}) or from the apical bath (P_{osm}^{ap}). In all these situations AQP2-transfected cells resulted in P_{osm} values significantly higher than those observed in cells not expressing AQP2. Hypotonic shocks, either apical or basolateral, resulted in similar P_{osm} values in WT-RCCD$_1$ cells ($P_{osm}^{bl} = P_{osm}^{ap}$) (Fig. 5*A*). Conversely, in AQP2-RCCD$_1$ cells, apical hypotonic shocks clearly increased P_{osm} values as compared with basolateral hypotonic shocks ($P_{osm}^{ap} > P_{osm}^{bl}$).

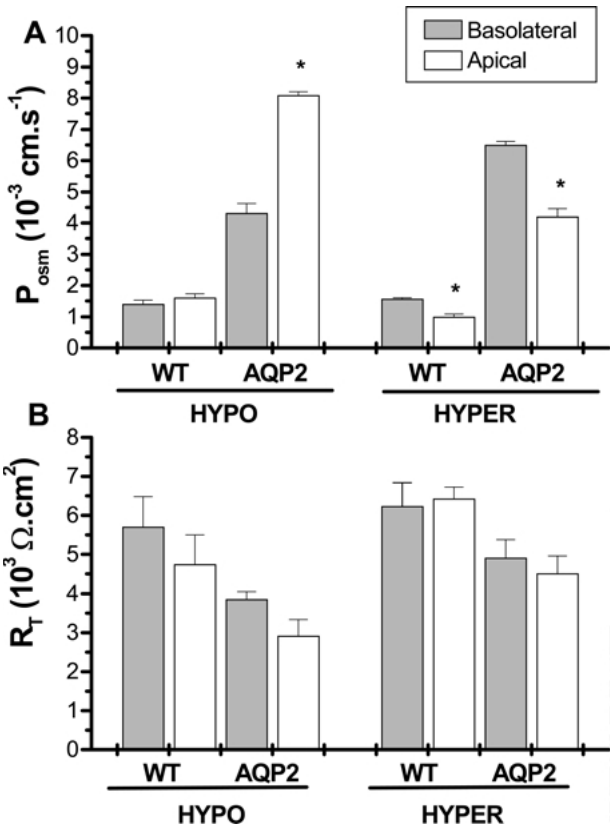

Fig. 5. Osmotic permeability and transepithelial resistance in WT-$RCCD_1$ and AQP2-$RCCD_1$ cells. (*A*) P_{osm} was evaluated, at steady state, after hyper- or hypotonic shock imposed either in the basolateral or apical bath. Values are mean ± SE of 6 to 8 independent experiments. $^*p < 0.001$ (basolateral vs. apical), Student's *t*-test for unpaired data. (*B*) Transepithelial resistance (R_T) was measured, at steady state, after hyper-or hypotonic shock imposed either in the basolateral or apical bath. Values are mean ± SE of 6 independent experiments.

These results suggest that hypotonic gradients only induce rectification in cells expressing AQP2.

On the other hand, basolateral hypertonicity induced higher P_{osm} values compared with those observed after apical hypertonicity ($P_{osm}^{bl} > P_{osm}^{ap}$) in both cell lines. Furthermore, the P_{osm}^{bl} / P_{osm}^{ap} relationship was identical in both cases (WT-$RCCD_1$ = 1.66 and AQP2-$RCCD_1$ = 1.56). These results indicate that, independently of AQP2 expression, basolateral solutions made hypertonic by adding an impermeable solute (100 mOsm mannitol) increased the water permeability of both $RCCD_1$ cell lines, thereby producing rectification of the osmotic water flow.

In order to determine whether these asymmetrical responses could be related to an osmotic contribution of the intercellular spaces (which can be decreased or increased by cell volume changes) the transepithelial resistance (R_T) was also evaluated. Fig. 5*B* shows R_T measured at the steady state, after hypo- or hypertonic challenges induced either from the basolateral or from the apical bath. It can be noted that in both cell lines no asymmetry in R_T was observed in either of these conditions. This result suggests that osmotic J_v rectification cannot be attributed to a shunt through the tight junction/intercellular space path.

Discussion

Biophysical Properties of Water Permeability in WT-$RCCD_1$ Versus AQP2-$RCCD_1$ Cells

In the present study, we have first characterized the water permeability properties of $RCCD_1$ cells either in the presence or not of aquaporin-2 (AQP2-$RCCD_1$ vs. WT-$RCCD_1$ cells). Our results show that transepithelial P_{osm} values in WT-$RCCD_1$ cells are in good agreement with those described for different collecting duct cell lines [26, 42], as well as for native CCD tubules before AVP stimulation [24, 34, 38].

In AQP2-$RCCD_1$ cells, which constitutively express AQP2 in the apical plasma membrane [17], transepithelial P_{osm} values were about 2- to 6-fold higher than in WT-$RCCD_1$ cells. Similarly, previous work in collecting duct AQP2-transfected cells, expressing AQP2 in intracellular vesicles, reported a 2- to 10-fold increase in P_{osm} after AVP or forskolin stimulation [26, 42]. Our results also demonstrated that P_{osm} was modulated after AVP stimulation and this response was dependent on cytoskeleton integrity. This observation allowed us to confirm that in addition to their constitutive AQP2 expression these cells are also able to incorporate functional aquaporins to the plasma membrane.

On the other hand, hydrostatic permeability (P_{hydr}) was not significantly different between WT-$RCCD_1$ and AQP2-$RCCD_1$ cells. P_{hydr} values for $RCCD_1$ cells, although higher than P_{osm}, are in good agreement with those previously reported by our laboratory and by others for renal epithelial cell lines grown on filters [30, 39]. The lack of difference between WT and AQP2-$RCCD_1$ cells in this parameter strongly suggests that a hydrostatic gradient mainly induces paracellular fluid movements, as previously reported in many systems [16, 32].

Altogether these results led us to propose that osmotic gradients, contrary to hydrostatic ones, mainly reflect transcellular water movements in $RCCD_1$ cells.

Asymmetrical Response to Osmotic Shocks

Our present experiments demonstrated that $RCCD_1$ cells responded differently to hypo- or hypertonic gradients. Following hypotonic shocks $RCCD_1$ cells only showed rectification of water flow in the

presence of apical AQP2. In contrast, in both WT and AQP2-RCCD$_1$ cells, P_{osm} values were significantly higher when basolateral tonicity was made hypertonic compared to that observed after apical hypertonicity ($P_{osm}^{bl} > P_{osm}^{ap}$).

A considerable number of previous studies have demonstrated osmotic rectification in many epithelia [5, 6, 25, 37]. However, this response has not yet been completely explained. Particularly, studies of isolated perfused cortical and medullary collecting tubules have demonstrated that institution of osmotic gradients decreases [19], increases [28] or has no effect [34] on transepithelial P_{osm}. It was proposed that these apparently disparate observations may be the result of species differences and nephron segment [20]. Nevertheless, interpretation of these results should be preceded by a careful search for uncontrolled asymmetrical forces imposed by the experimental design. It was previously reported that asymmetric osmotic water flow across a membrane in some epithelia could be produced by adjacent unstirred fluid layers, as discussed by Dainty [8]. However, it has been widely assumed that P_{osm} measurements are not limited by unstirred layer effects in the collecting duct and other tight epithelia [4, 24, 31, 35, 37, 38]. It has been also suggested that rectification of vasopressin-independent transepithelial water movement in the CCD exists because of asymmetry in paracellular permeability [37]. We found, however, that the steady-state transepithelial resistance of both cell lines, WT-RCCD$_1$ and AQP2-RCCD$_1$, was symmetrical after apical or basolateral increases or decreases in osmolality (Fig. 5*B*). These results are also in agreement with those observed in another cell line (LLC-PK1 cells) transfected with AQP2, which clearly showed that an important increase in P_{osm} can be associated with no significant changes in R_T [40]. Therefore, our observed asymmetry would not be due to paracellular transport or unstirred layer effects.

Other authors attributed asymmetrical water transport properties, in response to water flow in different directions, to changes in cell volume and/or cell morphology [10, 22, 38]. We have previously reported, in WT and AQP2-RCCD$_1$ cells, that osmotic stress induces patterned responses in activation of channels or transporters [17, 18]. We here suggest that osmotic-flow rectification is not a simple process but would rather depend on the asymmetrical modulation of, at least, one individual membrane permeability. Particularly, our central proposal is that membrane permeability would be a function of the osmolality it faces. How could osmolality contribute to this modulation? The simplest explanation is that a membrane could dynamically vary the osmotic permeability by changes in the number and/or activities of aquaporins, as previously reported [24, 27, 35]. Nevertheless, this proposal alone seems unlikely for WT-RCCD$_1$ cells, which do not express AQPs, although they also showed rectification of P_{osm} under hypertonic shocks. Then, at least in the absence of AQPs, further mechanisms must be involved to explain osmotic rectification. Previous observations indicated that osmomechanical stress can activate numerous membrane-associated events including activation of plasma membrane ion channels or transporters and intracellular signaling events [17, 33]. Hence, osmolality of the incubation medium and/or changes in volume of an osmo-sensitive compartment may modulate, within minutes, the intrinsic solute/ion membrane permeabilities. This modulation would increase/decrease the intracellular solute/ion content, building up local osmosis, which is a driving force that moves water. In this situation, water could move "actively" and a new component must be added to the initial osmotic (passive) flux (J_{osm}^{P}): the water-ion coupled (active) volume flux (J_{osm}^{A}). Then, the overall osmotic permeability will be: $P_{osm} = (J_{osm}^{P} + J_{osm}^{A}) / \Delta OSM$. The J_{osm}^{A} must not be misinterpreted as the "active water flux" observed in the absence of osmotic gradients ($\Delta Osm = 0$) since it is only activated by osmolality. If these mechanisms are either absent or not activated, J_{osm}^{P} would be the only component affecting P_{osm}, i.e., $P_{osm} = J_{osm}^{P} / \Delta Osm$. This possibility seems likely for WT-RCCD$_1$ cells, since our results showed that P_{osm} was partially dependent on the metabolic machinery of these cells (see Fig. 1*B*).

Therefore, the osmotic rectification observed in RCCD$_1$ cells could be explained in terms of asymmetrical activation or incorporation of AQPs and/or channels/transporters to the plasma membrane, via some mechanism triggered by osmolaiity. To test whether the modulation of individual membrane permeabilities by osmolaiity may explain our experimental results we have employed a very simple and semi-quantitative model. Assuming the simplest composite membrane system (2 cell membranes in series) for transcellular pathway, the overall P_{osm} for the epithelium is given by:

$$\frac{1}{P_{osm}} = \frac{1}{P_a} + \frac{1}{P_b}$$

Where P_a is the osmotic permeability of the apical membrane, and P_b is the osmotic permeability of the basolateral membrane [21]. The cytoplasm resistance was considered negligible. To satisfy the experimental asymmetries ($P_{osm}^{bl} \neq P_{osm}^{ap}$), we fitted P_{osm} in Eq. 1, by using Excel Solver linear programming, to all experimental values, assuming that at least the permeability of one individual membrane is a function of the osmolaiity it faces, while the other one is considered constant.

For hypotonic shocks, WT-RCCD$_1$ cells were not considered, since no rectification was observed ($P_{osm}^{ap} = P_{osm}^{bl}$). In AQP2-RCCD$_1$ cells we have

supposed that P_a would be affected by its neighborhood osmolality, while P_b would be independent of the osmolality it faces. This assumption is based upon our previous reports in AQP2-RCCD$_1$ cells, showing that K^+ channels, CFTR (rapid RVD mechanisms) and AQP2, are located at the apical membrane and RVD mechanisms are activated by apical hypotonicity [17]. Therefore, P_a could be modulated by hypotonicity, either regulating AQP2 or activating ion channels. Then we have two possible equations with three variables: P_b, the stationary P_a, when the osmotic shock is imposed on the basolateral side (P_a^{bl}) or on the apical side (P_a^{ap}). Various relative values can be assigned to each individual membrane permeability, but after eliminating all combinations giving no rectification or rectification, in contrast to that observed, only one possibility remains: $P_a^{ap} > P_a^{bl}$.

For hypertonic shocks, in WT-RCCD$_1$ cells, we have supposed that P_b would be dependent upon its neighborhood osmolality, while P_a was considered constant. These statements are based on both our previous studies demonstrating that in WT-RCCD$_1$ cells AQPs are not expressed [7], together with the present results showing P_{osm} decreases with metabolic inhibitors (i.e, oubain, KCN) under basolateral hypertonicity. Therefore, P_b is a good candidate to be modulated in response to its boundary hypertonicity, probably due to solute/ion channel/transporter osmotic activation. We have two equations with three variables: P_a, the stationary P_b when an osmotic shock was generated on the apical side (P_b^{ap}) or on the basolateral side (P_b^{bl}). As above, all combinations resulting in no rectification or in rectification but inverse to that observed, were eliminated and only one possibility remained: $P_b^{ap} < P_b^{bl}$. For AQP2-RCCD$_1$ cells, asymmetry can be explained just by AQP2 modulation and then two possibilities may account for the observed rectification: i) an increase in P_b due to AQP2 incorporation when basolateral osmolality rises ($P_b^{bl} > P_b^{ap}$) or ii) a decrease in P_a due to AQP2 retrieval when apical osmolality rises ($P_a^{ap} < P_a^{bl}$). The first possibility seems more probable, as several studies proposed that AQP2 can be up-regulated by factors including hypertonicity [35, 41]. Since in AQP2-RCCD$_1$ cells AQP2 is mainly found in the apical membrane, we can speculate that it could be targeted, by hypertonicity, to the basolateral surface because of sorting-pathway saturation as previously reported [3].

Simulated individual membrane permeabilities were obtained fitting P_{osm} in Eq. 1 to all experimental values. Results showed that, in AQP2-RCCD$_1$ cells, apical hypotonicity resulted in P_a^{ap} values about 7-fold higher than those obtained under basolateral hypotonicity (P_a^{bl}) ($P_a \times 10^{-4}$ cm $\cdot$ s^{-1}: 711 vs. 92). During hypertonic shocks, WT-RCCD$_1$ cells showed that P_b^{bl} was about 4.5-fold higher than P_b^{ap} ($P_b \times 10^{-4}$ cm $\cdot$ S^{-1}: 89 vs. 18). In AQP2-RCCD$_1$ cells, P_b^{bl} was about 2.5-fold higher than P_b^{ap} ($P_b \times 10^{-4}$ cm $\cdot$ s^{-1}: 221 vs. 87). Therefore, this simple model allows explaining the asymmetrical results in terms of an individual stationary-membrane permeability. which, in turn, may be dependent on the osmolality it faces. This membrane permeability modulation could be, indeed, the result of plasma membrane channel or transporter activation together with AQP insertion, affecting the overall osmotic response. Whether these solute/ion channels or transporters are mechanisms involved in cell volume regulation as well as their putative association with AQP modulation remains to be determined. Additional investigations into the relationship between fluid transport and signaling mechanisms involved in these processes would be of physiological relevance.

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