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Aquaporin 2-Increased Renal Cell Proliferation Is Associated With Cell Volume Regulation

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ABSTRACT

We have previously demonstrated that in renal cortical collecting duct cells ($RCCD_1$) the expression of the water channel Aquaporin 2 (AQP2) raises the rate of cell proliferation. In this study, we investigated the mechanisms involved in this process, focusing on the putative link between AQP2 expression, cell volume changes, and regulatory volume decrease activity (RVD). Two renal cell lines were used: WT-$RCCD_1$ (not expressing aquaporins) and AQP2-$RCCD_1$ (transfected with AQP2). Our results showed that when most $RCCD_1$ cells are in the G_1-phase (unsynchronized), the blockage of barium-sensitive K^+ channels implicated in rapid RVD inhibits cell proliferation only in AQP2-$RCCD_1$ cells. Though cells in the S-phase (synchronized) had a remarkable increase in size, this enhancement was higher and was accompanied by a significant down-regulation in the rapid RVD response only in AQP2-$RCCD_1$ cells. This decrease in the RVD activity did not correlate with changes in AQP2 function or expression, demonstrating that AQP2—besides increasing water permeability—would play some other role. These observations together with evidence implying a cell-sizing mechanism that shortens the cell cycle of large cells, let us to propose that during nutrient uptake, in early G_1, volume tends to increase but it may be efficiently regulated by an AQP2-dependent mechanism, inducing the rapid activation of RVD channels. This mechanism would be down-regulated when volume needs to be increased in order to proceed into the S-phase. Therefore, during cell cycle, a coordinated modulation of the RVD activity may contribute to accelerate proliferation of cells expressing AQP2. J. Cell. Biochem. 113: 3721–3729, 2012.

KEY WORDS: CELL PROLIFERATION; AQUAPORIN 2; CELL CYCLE; CELL VOLUME REGULATION; RENAL CELLS

Cell growth and proliferation are closely associated with changes in cell volume [Meyer et al., 1991; Lang et al., 2000, 2005]. In fact, previous reports indicate that factors that affect cell volume also have an effect on mechanisms that control cellular proliferation [Rouzaire-Dubois and Dubois, 1998; Kunzelmann, 2005]. It is well accepted that during progression through the cell cycle, cellular volume increases as a result of the accumulation of diverse substrates, protein synthesis, and DNA duplication [Kunzelmann, 2005]. As cells are assumed to have a volume set point which, if exceeded, leads to the activation of regulatory mechanisms; to enhance their volume, cells must inhibit their regulatory volume decrease mechanisms (RVD) and/or activate regulatory volume increase (RVI) mechanisms by readjusting their volume set point to a higher level. In line with this proposal, several reports demonstrate that, in many cell types, RVI mechanisms are up-regulated during cell growth [Lang et al., 1991, 1998; Ritter and Wöll, 1996] and that RVD capacity is actively modulated throughout the cell cycle [Chen et al., 2002; Wang et al., 2002]. In addition, the pharmacological blockage of some channels involved in the RVD response has shown to inhibit cell proliferation [Arcangeli et al., 2009]. Other reports, in tumor ras-oncogene-expressing cells, suggest that both RVD and RVI mechanisms are necessary for cell cycle progression, since the initial activation of channels involved in RVD triggers later on an intracellular signalization response that results in an RVI response [Lang et al., 1991; Ritter and Wöll, 1996]. Data have also proved that an active size threshold mechanism exists in the G_1-phase, which induces adjustment of the length of the following cell cycle, thus ensuring maintenance of a proper balance between growth and proliferation rates in vertebrates. However, the nature of this process is completely

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unknown [Dolznig et al., 2004]. Therefore, all current evidence indicates that cellular proliferation would require mechanisms for cell volume regulation, and thus the movement of ions and water. However, little is known regarding the sequence of events leading from cell volume changes to cell cycle progression. Moreover, despite the fact that water channels (aquaporins, AQPs) have been proposed to play a key role not only in the control of cell volume but also in cell proliferation and apoptosis [Jablonski et al., 2004, 2007; Ford et al., 2005; Galizia et al., 2008; Verkman, 2008; Rivarola et al., 2010], no studies have been carried out so far as to evaluate the putative role of AQPs throughout the cell cycle.

We have previously demonstrated in renal cortical collecting duct cells ($RCCD_1$) that the water channel AQP2 plays a key role in cell volume regulation after an osmotic swelling (RVD), by rapidly activating barium-sensitive K^+ channels [Ford et al., 2005]. In fact, the presence of a functional AQP2 is critical in the translocation of calcium channel transient receptor potential vanalloid 4 (TRPV4) to the plasmatic membrane, which in turn switches on a Ca^{2+}-activated K^+ channel that leads to solute and water efflux, thus eliciting a rapid RVD response [Ford et al., 2005; Galizia et al., 2008, 2012]. A functional interaction between AQP2 and RVD mechanisms would also explain the increase in the apoptotic volume decrease of cells expressing AQP2, as compared to those not expressing AQPs [Flamenco et al., 2009]. Furthermore, our group previously demonstrated that AQP2 expression enhances renal cell proliferation rate by decreasing the transit time through S- and G_2/M-phases of the cell cycle [Rivarola et al., 2010]. In the present study, we investigated the mechanisms that could account for the AQP2-increased renal cell proliferation. Particularly, we focused on the putative link between AQP2 expression, cell volume changes, and regulatory volume response (RVD) during cell cycle progression. For this purpose, we used two renal cell lines: one not expressing AQPs (WT-$RCCD_1$) and another one stably transfected with AQP2 (AQP2-$RCCD_1$) that constitutively expresses AQP2 in the apical plasma membrane [Capurro et al., 2001; Ford et al., 2005]. Our results showed that only in the presence of AQP2, the blockage of rapidly activated RVD mechanisms reduces cell proliferation by arresting cells in the G_1-phase. Cells in the S-phase had a remarkable increase in size but this enhancement was higher and was accompanied by a significant down-regulation in the rapid RVD response in AQP2-$RCCD_1$ cells. We propose that during cell cycle a coordinated modulation of RVD activity, in response to changes usually occurring in cell volume, may contribute to accelerate proliferation of cells expressing AQP2.

MATERIALS AND METHODS

CELL CULTURE

WT-$RCCD_1$ cells were grown in a modified-DMEM (DM) medium (Dulbecco's modified Eagle's medium/Ham's F-12, 1:1 v/v; 14 mM $NaHCO_3$, 2 mM glutamine; 50 nM dexamethasone; 30 nM sodium selenite; 5 μg/ml insulin; 5 μg/ml transferrin; 10 ng/ml epidermal growth factor; 50 nM tri-iodothyronine; 100 U/ml penicillin–streptomycin; 20 mM Hepes; pH 7.4) and 2% fetal bovine serum (Invitrogen, San Diego, CA) at 37°C in 5% CO_2/95% air atmosphere [Blot-Chabaud et al., 1996]. AQP2-$RCCD_1$ cells, stably transfected with cDNA coding for rat AQP2, were maintained in DM medium containing geneticin (200 μg/ml; Invitrogen) as previously reported [Ford et al., 2005]. All experiments were performed on WT- and AQP2-$RCCD_1$ cells between the 20^{th} and 40^{th} passages.

CELL CYCLE SYNCHRONIZATION

Cells were synchronized in the S-phase of the cell cycle by the double-block technique, using thymidine as an inhibitor of DNA synthesis [Harper, 2005]. Both WT- and AQP2-$RCCD_1$ cells in exponential growth were first exposed for 12 h to 2 mM thymidine and then released in fresh media for another 12 h, later followed by a second thymidine block for another 12 h. To determine the percentage of cells in each stage of the cell cycle, cells were stained with propidium iodide (PI; Sigma–Aldrich, St. Louis, MO) and the DNA content was measured by flow cytometry.

CELL GROWTH MEASUREMENT

For cell number counting WT- and AQP2-$RCCD_1$ cells were first seeded onto a 24-well plate with a density of 10×10^3 cells/ml, allowing them to attach for 24 h. After that, cells were harvested by trypsinization, resuspended in PBS-trypan blue to exclude non-viable cells, and counted in a Neubauer hemocytometer. To evaluate the participation of volume regulation channels in cell proliferation, cell number was estimated in the presence and the absence of specific inhibitors. Thus, cells were seeded, and, after giving them enough time to attach, they were incubated for 48 h in the presence of 0.2 mM $BaCl_2$ or 10 μM ruthenium red (RR; Sigma–Aldrich).

CELL PROLIFERATION AND CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

Cell cycle phases and hence the degree of synchronicity was assessed by flow cytometry. WT- and AQP2-$RCCD_1$ cells were grown either in normal conditions or in the presence of the cell cycle arrest agent thymidine. For flow cytometry analysis, cells were harvested by trypsin digestion, centrifuged, and washed with PBS. Cells ($\sim 1 \times 10^6$ cells) were then fixed by adding chilled 70% ethanol and later stored at 4°C. For PI staining, the fixed cells were washed twice with PBS and incubated in the presence of PI (50 μg/ml) and RNase A (1 mg/ml) in PBS for up to 4 h at 4°C in the dark. The fluorescence intensity of PI-stained individual nuclei was measured using a FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA). Cell debris was excluded from analysis by appropriately raising the forward scatter threshold. The cell fractions in sub-G_0- (apoptotic cells), G_1-, S-, and G_2/M-phases were quantified with WinMDI 2.9 software after exclusion of cell doublets (Joseph Trotter, The Scripps Research Institute, La Jolla, CA).

For cell proliferation analysis, cells were pulsed with 20 μM of the thymidine analog 5-Bromodeoxyuridine (BrdU; Sigma–Aldrich) for 2 h. Cells were harvested and fixed in 70% ethanol, as described above. BrdU content was analyzed after DNA denaturation with 2 N HCl and 0.1% Triton X-100 at room temperature, followed by neutralization with 0.1 M $Na_2B_4O_7$ buffer, pH 8.5. The cells were labeled using specific anti-BrdU monoclonal antibodies (Sigma–Aldrich) and Cy2-conjugated secondary antibodies (Jackson Immuno, Pennsylvania, PA). BrdU/DNA content was obtained

collecting Cy2 green fluorescence and analyzed with WinMDI 2.9 software.

CELL VOLUME MEASUREMENTS

WT- and AQP2-RCCD$_1$ cells were grown on glass coverslips and stained with the plasma membrane marker Alexa 488-conjugated wheat germ agglutinin (WGA; Molecular Probes, Life Technologies, Grand Island, NY). Then, cells were fixed in 3% *p*-formaldehyde for 30 min and nuclei were counterstained with Hoechst 33258 (Molecular Probes). The coverslips were mounted on glass microscope slides with Vectashield mounting media (Vector Laboratories Inc., Burlingame, CA). Images were acquired using a confocal microscope Olympus FluoView (FV1000). A z-stack consisting of series of 0.5 μm optical slices was taken from top to bottom of the cells. These two-dimensional slices were then three-dimensionally reconstructed and cell volume was estimated using Imaris 7.1.0 software (Bitplane, Zurich, Switzerland).

FLUORESCENCE VIDEOMICROSCOPY MEASUREMENTS OF REGULATORY VOLUME DECREASE (RVD) ACTIVITY AND OSMOTIC WATER PERMEABILITY (P_F)

Confluent WT- and AQP2-RCCD$_1$ cells grown on glass coverslips were mounted on a chamber and loaded with 2 μM 2′,7′-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM; Molecular Probes) for 45 min at 37°C. The chamber was then placed on the stage of a Nikon TE-200 epifluorescence inverted microscope (Nikon Plan Fluor 40× oil immersion objective lens) as previously described [Ford et al., 2005]. Fluorescence intensity was recorded by exciting BCECF at the isosbestic point (440 nm), where the fluorochrome is pH insensitive. Fluorescence data were acquired every 10 s using a charge coupled-device camera (Hamamatsu C4742-95) connected to a computer and the Metafluor acquisition program (Universal Imaging Corporation, PA). Cells were incubated in an isosmotic solution (311 ± 6 mOsM) containing (mM): 90 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 0.8 $MgSO_4$, 1 $MgCl_2$, 100 mannitol, 20 Hepes, and 5 glucose. Hypotonic solutions (214 ± 6 mOsM) were prepared by mannitol subtraction to obtain the desired osmolality, thus maintaining the ionic strength. Solutions osmolality were routinely measured in a pressure vapor osmometer (Vapro; Wescor, Logan, UT). All solutions were titrated to pH 7.40 using Tris (Sigma–Aldrich) and bubbled with atmospheric air.

Cell water volume was estimated with a procedure similar to the one described for retinal pigment epithelial cells [Hamann et al., 2002]. The change in cell water volume can be calculated as:

$$\frac{V}{V_0} = \frac{(F_t/F_0) - f_b}{1 - f_b}$$

where V is the cell water volume at time t; V_0 is V when t = 0; F_0 represents the signal obtained from a small region of the cell (pinhole) equilibrated with isotonic medium with an osmolality OsM_0; F_t is the fluorescence from the same region at time = t in a solution with an osmolality OsM_t and f_b is the relative background. This last parameter corresponds to the y intercept of a plot of F_t/F_0 versus OsM_0/OsM_t, which is the relative fluorescence when no osmolality change is performed.

RVD at t = 20 min, associated with the volumetric response of cells exposed to hypotonic medium, was calculated using the following equation:

$$RVD_{20} = \left[\frac{(V/V_0)_{max} - (V/V_0)_{20}}{(V/V_0)_{max} - 1}\right] \times 100$$

where $(V/V_0)_{max}$ is the maximal value of V/V_0 attained during hypotonic swelling (peak) and $(V/V_0)_{20}$ represents the value of V/V_0 observed at time = 20 min. RVD_{20} thus denotes the magnitude of volume regulation at time = 20 min with 100% RVD indicating complete volume regulation and 0% indicating no volume regulation.

The time course of V/V_0 for each experiment was fitted with a single exponential function. The osmotic water permeability coefficient (P_f) was calculated using the exponential time constant (τ) through the equation:

$$P_f = \frac{V_0}{\tau \times A \times \Delta OsM \times V_w}$$

where A is the cell surface area; V_0 is the initial cell water volume; ΔOsM is the osmotic gradient; and V_W is the partial molar volume of water (18 cm^3/mol).

CELL SURFACE BIOTINYLATION ASSAY AND WESTERN BLOT STUDIES

90–95% confluent WT- and AQP2-RCCD$_1$ cells were washed three times in cold PBS and cell surface biotinylation was performed with a Cell Surface Protein Isolation kit (Pierce, Rockford, IL) following the manufacturer's instructions. Briefly, the cells were rinsed with PBS and then biotynilated using EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 min on ice. After quenching, the cells were harvested and solubilized in Lysis Buffer (Pierce) and incubated for 30 min on ice. After centrifugation at 10,000*g* for 2 min at 4°C, the supernatant was added to Immobilized NeutrAvidin™ gel (Pierce) and incubated for 60 min at room temperature. After being washed, the biotinylated proteins were eluted in SDS–PAGE sample buffer containing 50 mM dithiothreitol. These plasma membrane proteins were subjected to 12% SDS–polyacrylamide minigels electrophoresis using the Tris–Tricine buffer system [Rivarola et al., 2010], and then transferred to nitrocellulose membranes (Mini Protean II; Bio-Rad, Hercules, CA). Blots were blocked with 1% BSA and then probed using either mouse polyclonal anti-β-actin (dilution 1:2,000, Sigma–Aldrich) or rabbit polyclonal anti-rat AQP2 (dilution 1:1,000) generated against a peptide corresponding to amino acids 250–271 of AQP2, conjugated with keyhole limpet hemocyanin [Shi et al., 2001]. After being rinsed with PBS-T, membranes were incubated with the appropriate secondary antibody (goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase, dilution 1:25,000; Santa Cruz Biotechnology, Santa Cruz, CA and Sigma–Aldrich, respectively) and visualized using the chemiluminescence method (SuperSignal Substrate; Pierce). Images were captured on a G:BOX (Syngene, Frederick, MD) and the bands corresponding to AQP2 or β-actin proteins were quantified by a densitometric analysis using Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD) and expressed as the AQP2/β-actin ratio.

STATISTICAL ANALYSES

Values are reported as mean ± SEM, and n is the number of experiments, which include at least a total of 40–70 cells each. Student's *t*-test for unpaired data was applied and $P < 0.05$ was considered statistically significant.

RESULTS

MODULATION OF RVD ACTIVITY AFFECTS CELL CYCLE PROGRESSION IN AQP2-EXPRESSING CELLS

We first analyzed whether the AQP2-increased cell proliferation that we have previously described [Rivarola et al., 2010] could be linked to a specific activation of RVD mechanisms. Having previously demonstrated that there is a calcium channel TRPV4-dependent rapid RVD activity, which is only observed in the presence of AQP2 [Galizia et al., 2012], we now pretreated $RCCD_1$ cells with RR, which is a general blocker of TRPV-type channels. Our results showed that 48 h of cell treatment with 10 μM RR did not affect WT-$RCCD_1$ cell growth but significantly reduced the number of AQP2-$RCCD_1$ cells to a level similar to that observed in WT cells (Fig. 1A). We then investigated, by using flow cytometry measurements of PI stained cells, the proportion of cells in each cell cycle phase after 48 h of treatment with control solution (vehicle) or with RR (Fig. 1B). Figure 1C shows the net effect of RR on the distribution of cells in each phase of the cell cycle. As shown, the pretreatment of cells with the blocker evoked an increase in the percentage of cells in G_1-phase, no changes in the S-phase, and a decrease in the percentage of cells in the G_2/M-phase. Note that even if RR apparently affects both cell lines, the percentage in which cells in the G_1-phase increases is significantly higher in AQP2-$RCCD_1$ cells (2% vs. 9%, WT and AQP2, respectively), indicating that the activation of calcium channels TRPV4 is necessary for cell proliferation. It is important to note that the decline in cell proliferation was not due to an increase in cell death since the rate of apoptosis was not affected by the blocker (Vehicle vs. RR; WT: 11.7 ± 3.8 vs. 9.0 ± 2.2; AQP2: 16.7 ± 5.3 vs. 16.1 ± 3.5; n = 6, N.S.).

Since TRPV4 translocation/activation is necessary to trigger the AQP2-dependent rapid activation of barium-sensitive K^+ channels as an RVD mechanism [Ford et al., 2005; Galizia et al., 2012], the next step was to investigate whether this response could be implicated in the observed proliferation differences between WT- and AQP2-$RCCD_1$ cells. We first evaluated, by a videomicroscopy fluorescence technique, the characteristic of the rapid RVD response of cells incubated for 48 h with or without 0.2 mM $BaCl_2$ using a hypotonic shock as a strategy (−100 mOsM mannitol). Figure 2A,B shows the time course of relative cell volume changes (V/V_0) in WT- and AQP2-$RCCD_1$ cells. Note that in WT-$RCCD_1$ cells there were no changes in the kinetics of V/V_0 after cell treatment with vehicle or with $BaCl_2$. In contrast, in AQP2-$RCCD_1$ cells, the blocker significantly inhibited the kinetics of V/V_0 (Fig. 2B). Accordingly, the RVD response was significantly reduced only in AQP2-$RCCD_1$ cells (Fig. 2C). It is also important to point out that the 48 h exposure to $BaCl_2$ did not affect water permeability (P_f) neither in WT- nor in AQP2-$RCCD_1$ cells (Fig. 2D).

The next step was to evaluate the consequences that this RVD inhibition may have on the rate of cell proliferation. Figure 3A shows that the inhibition of barium-sensitive K^+ channels affects the number of cells only in the presence of AQP2. Further studies assessed BrdU incorporation by flow cytometry analysis in order to evaluate the proliferation of growing cells (Fig. 3B). Results showed that the percentage of BrdU positive cells ($BrdU^+$) was higher in AQP2-expressing cells and was significantly reduced in the presence of $BaCl_2$, only in cells expressing AQP2. We then performed flow cytometry measurements of PI-stained cells to determine whether these disparities in cell growth could be due to differences in cell cycle progression. Figure 3C illustrates the proportion of cells in each cell cycle phase after 48 h of treatment with either control

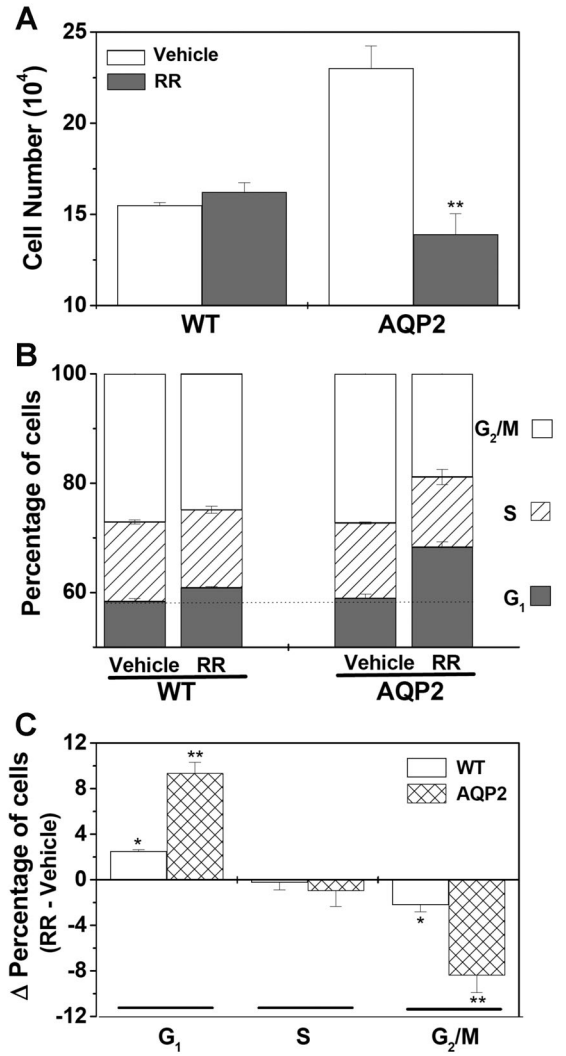

Fig. 1. Effect of ruthenium red (RR) on cell proliferation and cell cycle distribution in WT- and AQP2-$RCCD_1$ cells. Cells were treated for 48 h with control solution (Vehicle: DMSO) or with 10 μM RR. A: Cell number quantification using a hemocytometer. Values are presented as the mean ± SEM from n = 7 experiments, $^{**}P < 0.01$. B: Percentage of cells in G_1-, S-, and G_2/M-phases of the cell cycle, obtained by flow cytometry analysis of PI-stained cells and (C) net effect of RR treatment on the distribution of cells throughout the cell cycle, presented as the difference between RR- and vehicle-treated cells. Data represent the mean ± SEM from n = 6 experiments, $^{**}P < 0.01$.

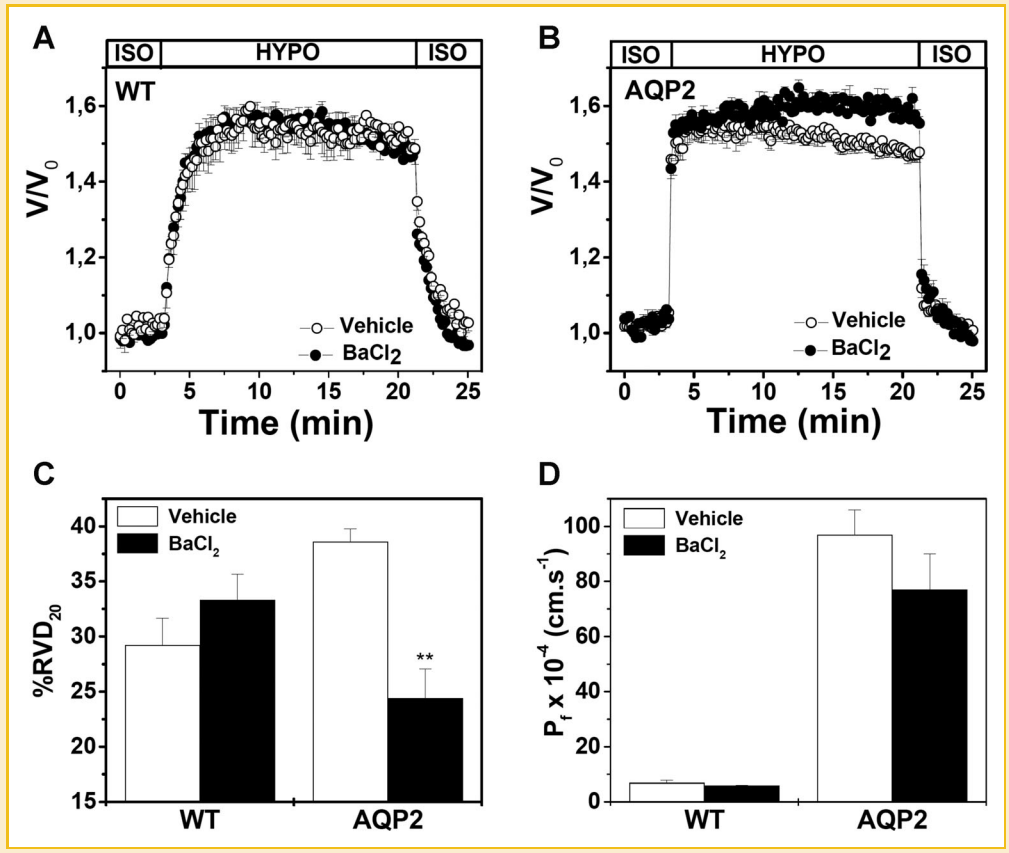

Fig. 2. Effect of $BaCl_2$ on the regulatory volume decrease activity and the osmotic water permeability in WT- and AQP2-$RCCD_1$ cells. Cells were treated for 48 h with control solution (Vehicle: PBS) or with 0.2 mM $BaCl_2$. A,B: Time course of relative volume changes (V/V_0) in response to a hypotonic shock in WT- and AQP2-$RCCD_1$ cells respectively, obtained with a videomicroscopy fluorescence technique. Hypotonic solution (HYPO) was achieved by substracting 100 mOsM mannitol (−100 mOsM mannitol) from the isotonic solution (ISO). C: Percentage of cell volume recovery at 20 min (% RVD_{20}) and (D) osmotic water permeability coefficient (P_f) calculated when the hypotonic shock (−100 mOsM mannitol) was applied in WT- and AQP2-$RCCD_1$ cells. Values denote the mean ± SEM from n = 4 experiments, $^{**}P < 0.01$.

solution (vehicle) or $BaCl_2$. The net effect of $BaCl_2$ on cell cycle progression is summarized in Figure 3D. These results indicate that, in the presence of $BaCl_2$, AQP2 expression increases the percentage of cells in G_1-phase and decreases the percentage of cells in G_2/M-phase, both relative to WT-$RCCD_1$ cells. This decline in cell proliferation is not explained by an increase in cell death since, on the contrary, $BaCl_2$ decreases the rate of apoptosis (Vehicle vs. $BaCl_2$; 14.4 ± 0.7 vs. 10.5 ± 1.3, $n = 7$, $P < 0.05$), as previously reported [Flamenco et al., 2009]. Together, these results suggest that the blockage of K^+ channels implicated in RVD inhibits cell proliferation by an arrest of the cell cycle and that this occurs only in AQP2-expressing cells.

RVD ACTIVITY IS DOWN-REGULATED IN THE S-PHASE OF THE CELL CYCLE IN AQP2-EXPRESSING CELLS

As stated above, evidence proved that cell cycle progression is closely related with cell volume changes and that the increment in cell volume during the G_1-phase allows cell progression into the S-phase [Dubois and Rouzaire-Dubois, 2004]. Therefore, to test our hypothesis that RVD activity must be modulated before entering the S-phase, synchronized and unsynchronized cells were used. The percentage of cells in the S-phase was increased by the double thymidine block technique (Fig. 4A,B). Figure 4C,D shows the effect of thymidine synchronization on cell volume, measured with confocal images after staining cell membranes with WGA. Results indicate that the double thymidine blockage was effective to enhance proportion of cells in the S-phase and to define that a significant increase in cell volume occurs in both cell lines. This cell size enhancement was higher in AQP2-expressing cells (76% vs. 92% WT- and AQP2-$RCCD_1$, respectively).

We further investigated the RVD response in both experimental conditions (Control vs. Thymidine) using the videomicroscopy fluorescence technique. Results shown in Figure 5A indicate that the rapid AQP2-dependent RVD mechanisms were down-regulated in AQP2-$RCCD_1$ synchronized cells and that no significant changes were observed in WT-$RCCD_1$ cells. However, the osmotic water permeability (P_f), which is significantly higher in AQP2-$RCCD_1$ cells compared to WT-$RCCD_1$ cells, was not affected in synchronized cells (Fig. 5B). Figure 5C shows Western blot assays of biotinylated plasma membrane samples, using a polyclonal anti-AQP2 antibody in synchronized and unsynchronized AQP2-$RCCD_1$ cells. The quantification of the relative bands showed no differences in AQP2 expression. All these results demonstrated that modulation of RVD activity does not correlate with changes in AQP2 function or

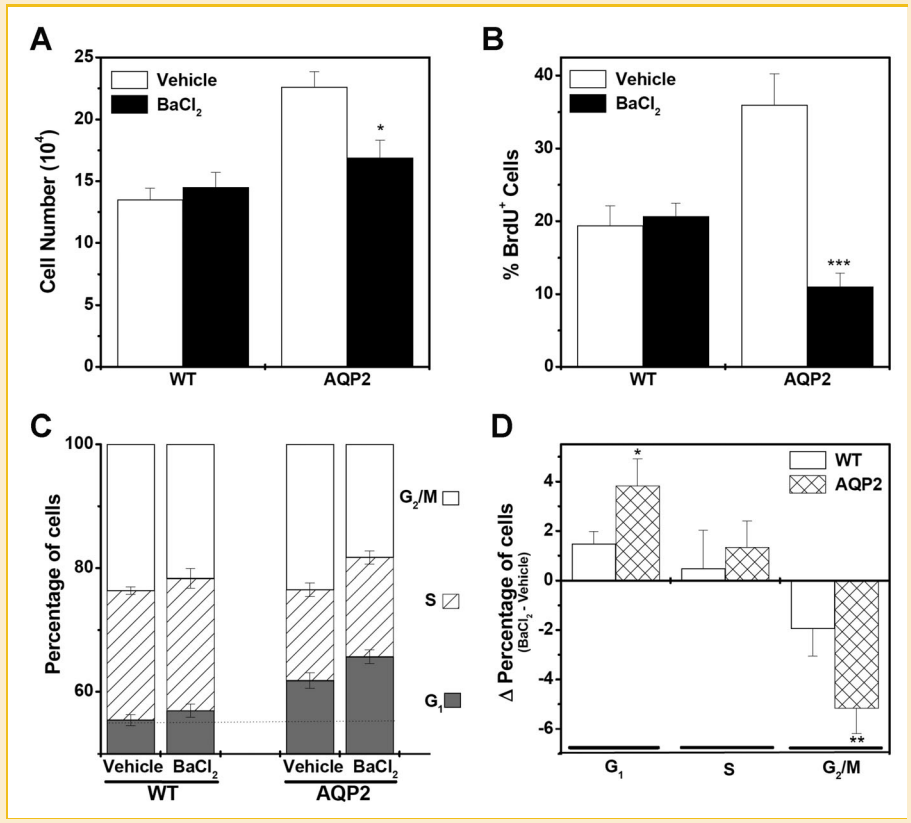

Fig. 3. Effect of $BaCl_2$ on cell proliferation and cell cycle distribution in WT- and AQP2-$RCCD_1$ cells. Cells were treated for 48 h with control solution (Vehicle: PBS) or with 0.2 mM $BaCl_2$. A: Cell number quantification using a hemocytometer and (B) percentage of $BrdU^+$ cells. Values are presented as the mean ± SEM, n = 4 experiments, $^{***}P < 0.001$. C: Percentage of cells in G_1-, S-, and G_2/M-phases of the cell cycle, obtained by flow cytometry analysis of PI-stained cells and (D) net effect of $BaCl_2$ treatment on the distribution of WT- and AQP2-$RCCD_1$ cells throughout the cell cycle, presented as the difference between treated and untreated cells. Data represent the mean ± SEM, n = 8 experiments, $^{**}P < 0.01$.

expression, thus supporting the idea that AQP2, besides increasing water permeability, would play some other role in the modulation of cell volume regulatory mechanisms.

DISCUSSION

Although there is consensus on accepting that the major role of AQPs is to increase plasma membrane water permeability, the use of different models—that express, or not, specific AQPs—provides new insights on previously unrecognized roles, such as cell migration, cell volume regulation, and apoptosis [Jablonski et al., 2004; Ford et al., 2005; Galizia et al., 2008; Verkman, 2008; Flamenco et al., 2009]. Nevertheless, AQPs participation in cell proliferation and cell cycle progression still remains relatively unclear. An increasing body of evidence proposes that the effect of AQPs expression on cell growth would not be a general phenomenon of all AQP-expressing cells, but may possibly depend on the cell type [Hoque et al., 2006; Kang et al., 2008; Verkman, 2008]. Our previous results showed that AQP2 expression in $RCCD_1$ accelerates proliferation rate by decreasing S- and G_2/M-phase transit times during cell cycle progression [Rivarola et al., 2010]. In the present study, the mechanisms behind AQP2-increased cell proliferation in this cell type were investigated for the first time.

Our results indicate that when most $RCCD_1$ cells are in the G_1-phase (unsynchronized), both indirect (RR) and direct ($BaCl_2$) blockage of volume-regulatory K^+ channels implicated in RVD inhibits cell proliferation only in AQP2-$RCCD_1$ cells, producing an arrest of the cell cycle. This decline in cell growth was not due to changes in cell viability, since the rate of apoptosis was not augmented. The observation that RVD mechanisms contribute to renal cell proliferation is in close agreement with previous studies performed in other cell types. These studies demonstrated that swelling-activated Cl^- or K^+ channels may play an important role in controlling cell cycle progression since channels are expressed in a cell cycle-dependent manner and their blockage affects the rate of cell proliferation [Rouzaire-Dubois et al., 2000; Nilius, 2001; Wang et al., 2004; Chen et al., 2007; Klausen et al., 2007; Tao et al., 2008]. Our data also proved that cells in the S-phase have a remarkable increase in size but this enhancement was higher and was accompanied by a significant drop in the rapid RVD activity in cells expressing AQP2. This evidence favors the idea that in the presence of AQP2 a coordinated modulation of RVD mechanisms may contribute to accelerate cell cycle.

In addition, we demonstrated that the down-regulation of AQP2-dependent RVD activity in the S-phase does not correlate with changes in AQP2 expression or with changes in plasma membrane

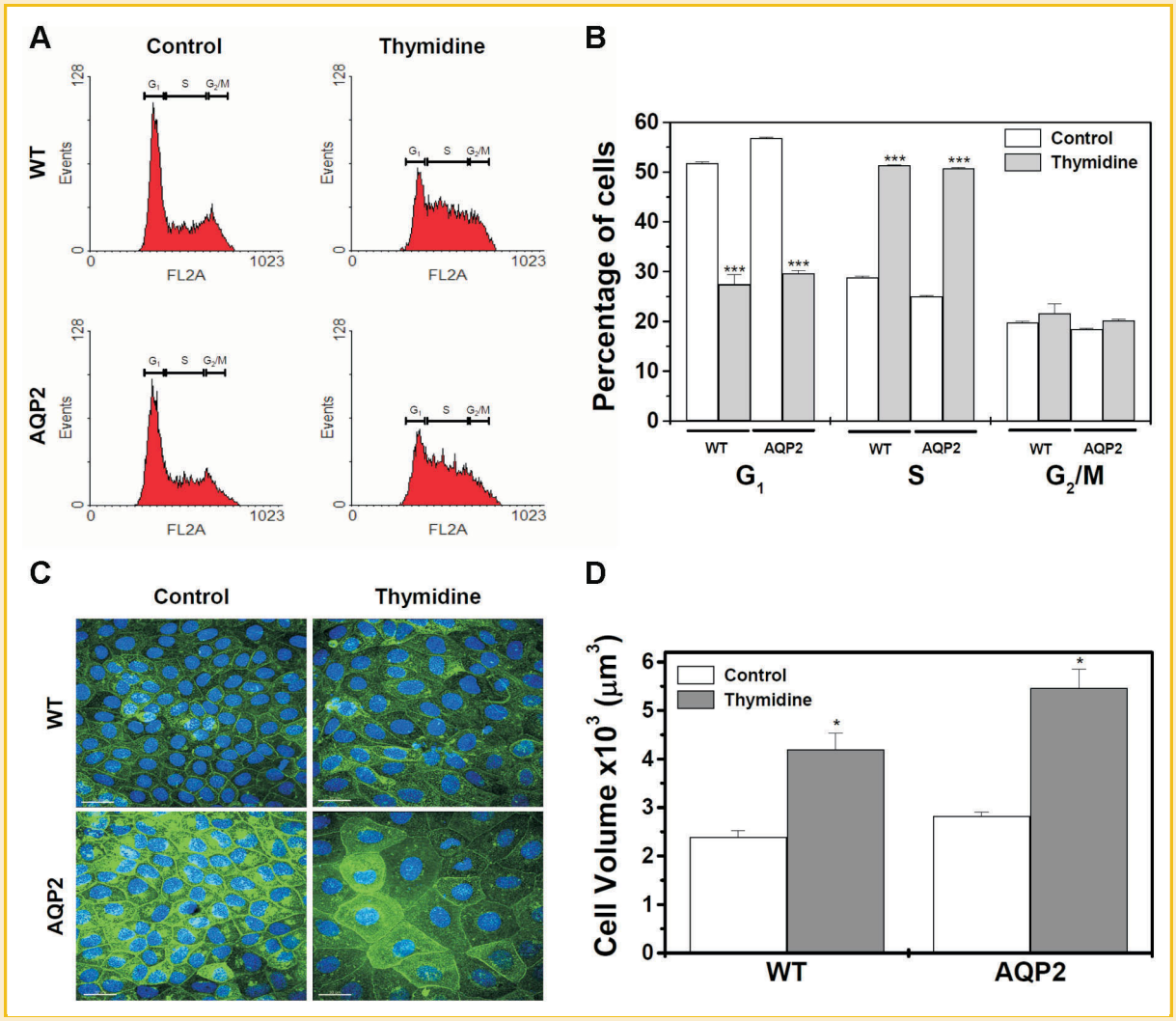

Fig. 4. Thymidine synchronization of WT- and AQP2-RCCD$_1$ cells. A: Typical flow cytometry profiles showing the distribution of cell cycle phases normally found in unsynchronized cells (Control) and the increase of the percentage of cells in the S-phase after a double thymidine block (Thymidine) both in WT- and AQP2-RCCD$_1$ cells. B: Percentage of WT- and AQP2-RCCD$_1$ cells in each cell cycle phase, either under control conditions or following treatment with thymidine, obtained by flow cytometry analysis. Data are presented as the mean ± SEM of four experiments, $^{***}P < 0.001$. C: Representative images of WT- and AQP2-RCCD$_1$ cells in control or synchronized conditions (Thymidine). Cells were stained with the plasma membrane marker WGA and nuclei were counterstained with Hoechst. Confocal z-stacks of images were taken from top to bottom of the cells and were analyzed with Imaris 7.1.0. These images illustrate the cell volume increase observed in both cell lines after thymidine synchronization. Scale bars: 30 μm. D: Cell volume values (μm^3) were obtained by analyzing three-dimensional reconstructions of the confocal images obtained along the z-axis using Imaris 7.1.0 software. Data are presented as the mean ± SEM for 40 cells from three experiments, $^{***}P < 0.001$.

water permeability. The lack of AQP2 modulation during the cell cycle could be simply due to the fact that this water channel is not native, but stably transfected instead. However, we have shown previously that in RCCD$_1$ cells AQP2 expression can be largely modulated in some conditions, such as alkalosis [Rivarola et al., 2010]. Hence, it is likely that, at least for this cell type, changes in AQP2 expression/function during the cell cycle are not key factors to accelerate cell proliferation, supporting the hypothesis that AQP2 plays some other role. The absence of changes in AQP2 expression between G$_1$- and S-phases in RCCD$_1$ cells contrasts with previous reports for other AQPs in other cell types. In fact, it was shown that AQP1 mRNA and protein levels in A5 cells or AQP4 expression in astrocytes were maximal when most cells were in G$_1$-phase [Delporte et al., 1996; Yoneda et al., 2001]. However, as functional assays were not performed in those studies, the question if such variation in AQPs expression reflects a primary role in modulation of cell growth remains uncertain.

Our results together with evidence implying a cell-sizing mechanism that shortens the cell cycle for larger cells [Tzur et al., 2009]; let us to propose that AQP2-dependent cell volume control may explain the decrease in cell cycle length observed in the presence of this water channel. In RCCD$_1$ cells, as in other cell types, early in the G$_1$-phase volume tends to increase by the intracellular accumulation of substrates. However, if AQP2 is expressed, a rapid activation of RVD mechanisms is triggered—allowing an efficient control of cell volume—to maintain proper concentrations of

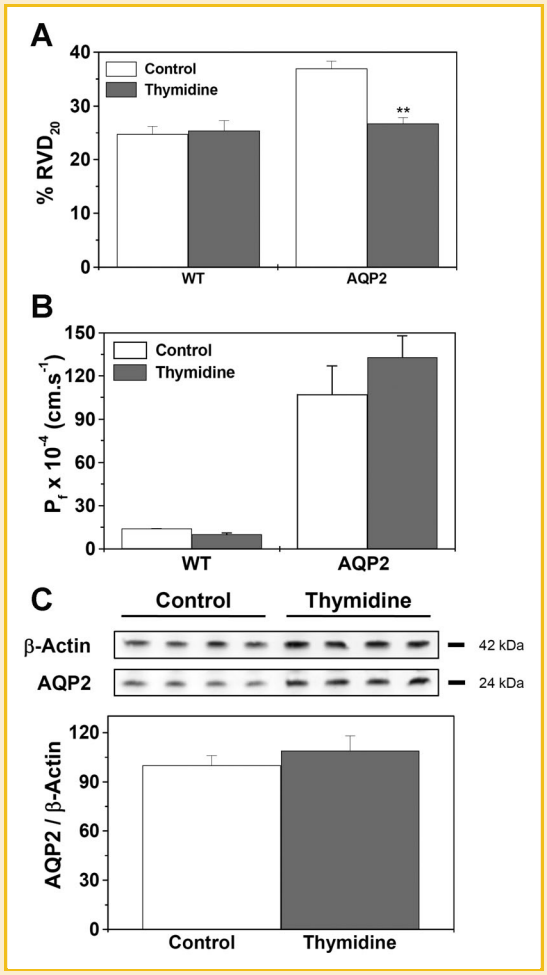

Fig. 5. Regulatory volume decrease activity, AQP2 expression and function in S-phase-synchronized WT- and AQP2-RCCD$_1$ cells. A: Percentage of cell volume recovery at 20 min (% RVD$_{20}$) obtained with a videomicroscopy fluorescence technique after the application of a hypotonic shock (−100 mOsM mannitol) in synchronized (Thymidine) or unsynchronized (Control) WT- and AQP2-RCCD$_1$ cells. Values represent the mean ± SEM of n = 4 experiments, $^{**}P < 0.01$. B: Osmotic water permeability coefficient (P_f) estimated using a videomicroscopy fluorescence technique when a hypotonic shock (−100 mOsM mannitol) was applied in synchronized (Thymidine) and unsynchronized (Control) WT- and AQP2-RCCD$_1$ cells. Values represent the mean ± SEM of n = 4 experiments. C: Representative immunoblots of biotinylated plasma membrane samples from AQP2-RCCD$_1$ synchronized (Thymidine) and unsynchronized (Control) cells. AQP2 was detected as a 24 kDa band. β-Actin (42 kDa) was used as an internal loading control. For densitometric quantification of immunoblots, samples from treated cells were run on each gel with corresponding controls. The results were normalized for β-actin density. The amount of AQP2 in synchronized cells was calculated as the percentage of the mean control value. Data represent the mean ± SEM of four independent experiments.

important factors needed for the regulation of the cell cycle progression. At some point, AQP2 cell volume control would also facilitates the down-regulation of RVD activity to let cells increase their volume in order to proceed into the S-phase. Thus, the volume of cells arriving into the S-phase will define the transit time through this phase.

How can cells expressing AQP2 lead to the rapid activation of specific volume-regulatory mechanisms that are not activated in the absence of this water channel? Our group and others propose that the AQPs may function as a component of a sensing-complex required to activate RVD [Hill et al., 2004; Ford et al., 2005; Fischbarg, 2006]. Therefore, it is tempting to speculate that during the G_1-phase any signal, such as changes in intracellular ionic strength, cytosolic pH, membrane voltage, Ca^{2+} levels, and/or cell volume, would be efficiently sensed by this complex, inducing the rapid modulation of RVD channels. However, it cannot be discarded that in order to increase their volume RVD mechanisms may be overtaken by RVI ones as described in previous reports [Lang et al., 1991; Ritter and Wöll, 1996]. We have preliminary data indicating that AQP2-expressing cells have an increased in the levels of NHE2 isoform of the Na^+/H^+ exchanger, a mechanism involved in RVI response, and that its blockage inhibits proliferation only in AQP2-RCCD$_1$ cells (Rivarola et al., unpublished results). Further experimental efforts are needed to address this complex interplay between AQPs and RVD/RVI activity at each phase of the cell cycle. Clarify the mechanisms governing cell volume control by AQP2 would be very important in learning about correct cellular function as well as about diseases associated with failure in AQP2 expression and alterations in cell proliferation as in the case of Insipidus Nephrogenic Diabetes induced by chronic lithium treatment.

REFERENCES

Arcangeli A, Crociani O, Lastraioli E, Masi A, Pillozzi S, Becchetti A. 2009. Targeting ion channels in cancer: A novel frontier in antineoplastic therapy. Curr Med Chem 16(1):66–93.

Blot-Chabaud M, Laplace M, Cluzeaud F, Capurro C, Cassingena R, Vandewalle A, Farman N, Bonvalet JP. 1996. Characteristics of a rat cortical collecting duct cell line that maintains high transepithelial resistance. Kidney Int 50(2):367–376.

Capurro C, Rivarola V, Kierbel A, Escoubet B, Farman N, Blot-Chabaud M, Parisi M. 2001. Vasopressin regulates water flow in a rat cortical collecting duct cell line not containing known aquaporins. J Membr Biol 179(1):63–70.

Chen L, Wang L, Zhu L, Nie S, Zhang J, Zhong P, Cai B, Luo H, Jacob TJ. 2002. Cell cycle-dependent expression of volume-activated chloride currents in nasopharyngeal carcinoma cells. Am J Physiol Cell Physiol 283(4):C1313–C1323.

Chen LX, Zhu LY, Jacob TJ, Wang LW. 2007. Roles of volume-activated Cl^- currents and regulatory volume decrease in the cell cycle and proliferation in nasopharyngeal carcinoma cells. Cell Prolif 40(2):253–267.

Delporte C, Chen ZJ, Baum BJ. 1996. Aquaporin 1 expression during the cell cycle in A5 cells. Biochem Biophys Res Commun 228(2):223–228.

Dolznig H, Grebien F, Sauer T, Beug H, Mullner EW. 2004. Evidence for a size-sensing mechanism in animal cells. Nat Cell Biol 6(9):899–905.

Dubois JM, Rouzaire-Dubois B. 2004. The influence of cell volume changes on tumour cell proliferation. Eur Biophys J 33(3):227–232.

Fischbarg J. 2006. Aquaporins and fluid transport: An evolving relationship. Cell Mol Biol (Noisy-le-grand) 52(7):28–33.

Flamenco P, Galizia L, Rivarola V, Fernandez J, Ford P, Capurro C. 2009. Role of AQP2 during apoptosis in cortical collecting duct cells. Biol Cell 101(4): 237–250.

Ford P, Rivarola V, Chara O, Blot-Chabaud M, Cluzeaud F, Farman N, Parisi M, Capurro C. 2005. Volume regulation in cortical collecting duct cells: Role of AQP2. Biol Cell 97(9):687–697.

Galizia L, Flamenco MP, Rivarola V, Capurro C, Ford P. 2008. Role of AQP2 in activation of calcium entry by hypotonicity: Implications in cell volume regulation. Am J Physiol Renal Physiol 294(3):F582–F590.

Galizia L, Pizzoni A, Fernandez J, Rivarola V, Capurro C, Ford P. 2012. Functional interaction between AQP2 and TRPV4 in renal cells. J Cell Biochem 113(2):580–589.

Hamann S, Kiilgaard J, Litman T, Alvarez-Leefmans F, Winther BR, Zeuthen T. 2002. Measurement of cell volume changes by fluorescence self-quenching. J Fluoresc 12(2):139–145.

Harper JV. 2005. Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol 296:157–166.

Hill AE, Shachar-Hill B, Shachar-Hill Y. 2004. What are aquaporins for? J Membr Biol 197(1):1–32.

Hoque MO, Soria JC, Woo J, Lee T, Lee J, Jang SJ, Upadhyay S, Trink B, Monitto C, Desmaze C, Mao L, Sidransky D, Moon C. 2006. Aquaporin 1 is overexpressed in lung cancer and stimulates NIH-3T3 cell proliferation and anchorage-independent growth. Am J Pathol 168(4):1345–1353.

Jablonski EM, Webb AN, McConnell NA, Riley MC, Hughes FM, Jr. 2004. Plasma membrane aquaporin activity can affect the rate of apoptosis but is inhibited after apoptotic volume decrease. Am J Physiol Cell Physiol 286(4):C975–C985.

Jablonski EM, Mattocks MA, Sokolov E, Koniaris LG, Hughes FM, Jr., Fausto N, Pierce RH, McKillop IH. 2007. Decreased aquaporin expression leads to increased resistance to apoptosis in hepatocellular carcinoma. Cancer Lett 250(1):36–46.

Kang SK, Chae YK, Woo J, Kim MS, Park JC, Lee J, Soria JC, Jang SJ, Sidransky D, Moon C. 2008. Role of human aquaporin 5 in colorectal carcinogenesis. Am J Pathol 173(2):518–525.

Klausen TK, Bergdahl A, Hougaard C, Christophersen P, Pedersen SF, Hoffmann EK. 2007. Cell cycle-dependent activity of the volume- and Ca^{2+}-activated anion currents in Ehrlich lettre ascites cells. J Cell Physiol 210(3):831–842.

Kunzelmann K. 2005. Ion channels and cancer. J Membr Biol 205(3):159–173.

Lang F, Friedrich F, Kahn E, Woll E, Hammerer M, Waldegger S, Maly K, Grunicke H. 1991. Bradykinin-induced oscillations of cell membrane potential in cells expressing the Ha-ras oncogene. J Biol Chem 266(8):4938–4942.

Lang F, Lepple-Wienhues A, Paulmichl M, Szabó I, Siemen D, Gulbins E. 1998. Ion channels, cell volume, and apoptotic cell death. Cell Physiol Biochem 8(6):285–292.

Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tanneur V, Lepple-Wienhues A, Szabo I, Gulbins E. 2000. Cell volume in the regulation of cell proliferation and apoptotic cell death. Cell Physiol Biochem 10(5–6):417–428.

Lang F, Foller M, Lang KS, Lang PA, Ritter M, Gulbins E, Vereninov A, Huber SM. 2005. Ion channels in cell proliferation and apoptotic cell death. J Membr Biol 205(3):147–157.

Meyer M, Maly K, Uberall F, Hoflacher J, Grunicke H. 1991. Stimulation of K^+ transport systems by Ha-ras. J Biol Chem 266(13):8230–8235.

Nilius B. 2001. Chloride channels go cell cycling. J Physiol 532(Pt 3):581.

Ritter M, Wöll E. 1996. Modification of cellular ion transport by the Ha-ras oncogene: Steps towards malignant transformation. Cell Physiol Biochem 6(5):245–270.

Rivarola V, Flamenco P, Melamud L, Galizia L, Ford P, Capurro C. 2010. Adaptation to alkalosis induces cell cycle delay and apoptosis in cortical collecting duct cells: Role of aquaporin-2. J Cell Physiol 224(2):405–413.

Rouzaire-Dubois B, Dubois JM. 1998. K^+ channel block-induced mammalian neuroblastoma cell swelling: A possible mechanism to influence proliferation. J Physiol 510(Pt 1):93–102.

Rouzaire-Dubois B, Milandri JB, Bostel S, Dubois JM. 2000. Control of cell proliferation by cell volume alterations in rat C6 glioma cells. Pflugers Arch 440(6):881–888.

Shi H, Levy-Holzman R, Cluzeaud F, Farman N, Garty H. 2001. Membrane topology and immunolocalization of CHIF in kidney and intestine. Am J Physiol Renal Physiol 280(3):F505–F512.

Tao R, Lau CP, Tse HF, Li GR. 2008. Regulation of cell proliferation by intermediate-conductance Ca^{2+}-activated potassium and volume-sensitive chloride channels in mouse mesenchymal stem cells. Am J Physiol Cell Physiol 295(5):C1409–C1416.

Tzur A, Kafri R, LeBleu VS, Lahav G, Kirschner MW. 2009. Cell growth and size homeostasis in proliferating animal cells. Science 325(5937):167–171.

Verkman AS. 2008. Mammalian aquaporins: Diverse physiological roles and potential clinical significance. Expert Rev Mol Med 10:e13.

Wang L, Chen L, Zhu L, Rawle M, Nie S, Zhang J, Ping Z, Kangrong C, Jacob TJ. 2002. Regulatory volume decrease is actively modulated during the cell cycle. J Cell Physiol 193(1):110–119.

Wang LW, Chen LX, Jacob T. 2004. ClC-3 expression in the cell cycle of nasopharyngeal carcinoma cells. Sheng Li Xue Bao 56(2):230–236.

Yoneda K, Yamamoto N, Asai K, Sobue K, Fujita Y, Fujita M, Mase M, Yamada K, Nakanishi M, Tada T, Miura Y, Kato T. 2001. Regulation of aquaporin-4 expression in astrocytes. Brain Res Mol Brain Res 89(1–2):94–102.