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Functional Interaction Between AQP2 and TRPV4 in Renal Cells

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ABSTRACT

We have previously demonstrated that renal cortical collecting duct cells ($RCCD_1$), responded to hypotonic stress with a rapid activation of regulatory volume decrease (RVD) mechanisms. This process requires the presence of the water channel AQP2 and calcium influx, opening the question about the molecular identity of this calcium entry path. Since the calcium permeable nonselective cation channel TRPV4 plays a crucial role in the response to mechanical and osmotic perturbations in a wide range of cell types, the aim of this work was to test the hypothesis that the increase in intracellular calcium concentration and the subsequent rapid RVD, only observed in the presence of AQP2, could be due to a specific activation of TRPV4. We evaluated the expression and function of TRPV4 channels and their contribution to RVD in WT-$RCCD_1$ (not expressing aquaporins) and in AQP2-$RCCD_1$ (transfected with AQP2) cells. Our results demonstrated that both cell lines endogenously express functional TRPV4, however, a large activation of the channel by hypotonicity only occurs in cells that express AQP2. Blocking of TRPV4 by ruthenium red abolished calcium influx as well as RVD, identifying TRPV4 as a necessary component in volume regulation. Even more, this process is dependent on the translocation of TRPV4 to the plasma membrane. Our data provide evidence of a novel association between TRPV4 and AQP2 that is involved in the activation of TRPV4 by hypotonicity and regulation of cellular response to the osmotic stress, suggesting that both proteins are assembled in a signaling complex that responds to anisosmotic conditions. J. Cell. Biochem. 113: 580–589, 2012.

KEY WORDS: AQUAPORIN 2; TRANSIENT RECEPTOR POTENTIAL VANILLOID 4; CELL VOLUME REGULATION; INTRACELLULAR CALCIUM

Many cell types have evolved specialized mechanisms of volume regulation to counteract damage induced by either cell swelling or shrinking. Under hypotonic conditions, cells first respond by swelling and second by initiating a mechanism that allows them to recover their original volume, this mechanism is called regulatory volume decrease (RVD) [O'Neill, 1999; Okada et al., 2001; Lang, 2007]. Essentially all cells respond to hypotonic swelling with this complex mechanism but the response is usually incomplete probably due to the fact that a new steady-state volume is achieved [O'Neill, 1999]. RVD depends on the release of inorganic and organic osmolytes that reverse the osmotic gradient and direction of water flow [Okada et al., 2001; Strange, 2004; Lang, 2007]. A number of ion channels and transporters were identified as the pathways for volume-regulatory ionic flux [Lang, 2007], however, the underlying mechanism that senses the change in osmolarity and/or cell volume to initiate volume regulation is not yet completely understood.

We have previously demonstrated, in a rat cortical collecting duct cell line ($RCCD_1$) which exhibits many major functional properties of the cortical collecting duct (CCD) [Blot-Chabaud et al., 1996; Djelidi et al., 1997] that the presence of the water channel AQP2 in the apical membrane was crucial for a rapid activation of RVD [Ford et al., 2005]. We also established that this RVD is related to the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and to barium-sensitive potassium channels [Ford et al., 2005] causing the exit of K^+, HCO_3^-, and Cl^- from the cells. In a more recent work we demonstrated that the rapid activation of these RVD mechanisms is dependent of an increase in

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the intracellular calcium concentration ($[Ca^{2+}]_i$) that only occurs in the presence of AQP2 [Galizia et al., 2008]. In fact, wild-type cells not expressing AQP2 (WT-$RCCD_1$) showed a diminutive increase in $[Ca^{2+}]_i$ and were incapable of producing a rapid activation of RVD. In AQP2-$RCCD_1$ cells the increase in Ca^{2+} was due to extracellular Ca^{2+} entry and to the release from intracellular stores. These two sources of calcium may act synergistically to produce the level of $[Ca^{2+}]$ increase necessary to turn on a Ca^{2+}-activated K^+ channel leading to solute and water efflux and consequently volume decrease. Therefore, these results probably indicate that AQP2, in concordance with other proteins, may form part of the cellular device for volume sensing and regulation. However, the molecular identity of the Ca^{2+} entry path involved in these responses remains to be investigated.

In recent years, it has become increasingly apparent that Ca^{2+} influx via transient receptor potential (TRP) channels plays a crucial role in the response to mechanical and osmotic perturbations in a wide range of cell types [Pedersen et al., 2005; Pedersen and Nilius, 2007]. Among the mammalian TRP channels, a role in volume regulation has been documented most thoroughly for the calcium permeable nonselective cation channel TRPV4 (transient receptor potential vanilloid 4). Importantly, activation of TRPV4 by cell swelling appears to be modulated by protein–protein interactions [Pedersen and Nilius, 2007]. In particular a few works propose an association between TRPV4 and some members of the AQPs family. In salivary gland epithelial it has been demonstrated that AQP5 is required for the activation of TRPV4 which increases intracellular Ca^{2+}, and that both proteins are assembled in a signaling complex that controls RVD [Liu et al., 2006]. In a recent study, Benfenati et al. [2011] found that a complex containing AQP4 and TRPV4 is essential for RVD in astrocytes. Co-expression of TRPV4 and AQP2 has been previously described in the human inner ear [Taguchi et al., 2007]. Therefore, the association between TRPV4 and AQPs in controlling RVD is beginning to be studied. It is important to identify whether this kind of interaction exists between AQP2 and TRPV4 in the kidney CCD cells, primarily effectors for systemic osmoregulation.

The aim of this work was to test the hypothesis that the increase in $[Ca^{2+}]_i$ and the subsequent rapid RVD only observed in the presence of AQP2 in renal CCD cells, could be due to a specific activation of a TRPV family member. TRPV4 is a good candidate to be investigated because it is osmosensible, it is expressed in kidney and, more interesting, it has been described to interact with AQP4 and AQP5 in other systems.

Using a fluorescent probe technique, we studied $[Ca^{2+}]_i$ and cell volume changes in response to a hypotonic shock in two renal cell lines, one not expressing aquaporins (WT-$RCCD_1$) [Capurro et al., 2001] and another stably transfected with AQP2 (AQP2-$RCCD_1$) that constitutively expresses AQP2 in the apical plasma membrane [Ford et al., 2005]. We have also studied the immunolocalization of TRPV4 during hypotonicity. The data here presented demonstrate that, in these cells, the presence of AQP2 is crucial for the translocation of TRPV4 to the plasma membrane, a process that seems to be necessary to control cell volume regulation under a hypotonic challenge.

MATERIALS AND METHODS

CELL CULTURE

WT-$RCCD_1$ cells were grown in modified DM medium (Dulbecco's modified Eagle's medium/Ham's F-12, 1:1 v/v, 14 mM $NaHCO_3$, 3.2 mM glutamine; 5×10^{-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 U/ml penicilin–streptomycin; 20 mM HEPES; pH 7.4) and 2% fetal bovine serum (FBS) (Gibco BRL) [Blot-Chabaud et al., 1996].

AQP2-$RCCD_1$ cells, stably transfected with cDNA coding for rat AQP2, were maintained in DM medium containing Geneticin (400 μg/ml, Life Technologies, Inc.) as previously reported [Ford et al., 2005].

All experiments were performed on confluent cells, between the 20th and 40th passages, grown on coverslips during 2 or 3 days.

CELL VOLUME CHANGES

We have measured cell volume in confluent WT and AQP2-$RCCD_1$ cells grown on coverslips using a fluorescent probe technique. The use of fluorescent dyes to monitor cell volume was earlier described by other authors [Crowe et al., 1995; Weinlich et al., 1998; Hamann et al., 2002]. We have previously tested in $RCCD_1$ cells the optimal conditions to collect fluorescence (F) [Ford et al., 2005]. Briefly, we found that the best condition was to collect F from a small circular region (pinhole) of 3–1% of the total area of the cell, localized in the periphery. Fluorescence from periphery was inversely proportional to the external osmolality and showed a linear correlation with the relative external osmolality [Ford et al., 2005]. For thin optical section through the cell a Nikon SFluor 40× nA 1.3 oil immersion objective lens was used. 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 30 min at 37°C. The chamber was then placed on the stage of a Nikon TE-200 epifluorescence inverted microscope. Cells were subsequently bathed at 20°C in dye-free solution for at least 15 min before the experiment. During experiments bathing solution was exchanged by aspirating the media and adding new media. This fluid exchange was completed within 5 s. Fluorescence intensity was recorded by exciting BCECF at the isosbestic point (440 nm HBW: 10 nm), where the fluorochrome is pH insensitive.

Fluorescence data were acquired every 10 s by use of a charge coupled device camera (Hamamatsu C4742-95) connected to a computer and the Metafluor acquisition program (Universal Imaging Corporation, PA). The procedure to estimate cell water volume was previously described [Hamann et al., 2002; Ford et al., 2005]. 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 epithelial 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 having an osmolality OsM_0; F_t is the fluorescence from the same region at time = t in a solution having an osmolality OsM_t and f_b is the relative background. This 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.

CELL VOLUME REGULATION

The RVD at 20 min, associated with the volumetric response of cells exposed to hypotonic medium was calculated by the use of 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 20 min. RVD_{20} thus denotes the magnitude of volume regulation at time 20 min, with 100% RVD indicating complete volume regulation and 0% RVD indicating no volume regulation.

INTRACELLULAR Ca^{2+} MEASUREMENT

WT- and AQP2-$RCCD_1$ cells were incubated in 10 μM Fura 2-AM (Molecular Probes, Inc.) for 60 min at room temperature and then washed to remove the excess of dye. To prevent dye compartmentalization upon loading, Pluronic F127 (0.2%) (Molecular Probes, Inc.) was used to dissolve the Fura 2-AM dye. The coverslips were again incubated in the experimental buffer for 15 min before the experiments. Fura 2 signal fluorescence was stimulated by dual-wavelength excitation at 340 and 380 nm. A 510 emission filter was used to collect Fura 2 signals at 10 s intervals. Ratios between the fluorescence intensity stimulated by 340/380 nm excitation were calculated.

Intracellular Ca^{2+} concentration was calibrated from maximum and minimum Fura 2 signals at the end of each experiment. Specifically, the bath solution was exchanged to 3.5 μM of the Ca^{2+} ionophore ionomycin (Sigma–Aldrich, St. Louis, MO) with 1 mM Ca^{2+} in the experimental buffer to establish maximum Fura 2 signals and also to Ca^{2+}-free experimental buffer with 100 μM EGTA (Sigma–Aldrich) and 3.5 μM of ionomycin to establish minimum Fura 2 signals. $[Ca^{2+}]_i$ was calculated as was previously reported [Grynkiewicz et al., 1985]

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)F$$

where K_d is the dissociation constant of Fura 2, R is the actual ratio of intensities at excitation wavelengths 340 and 380 nm, R_{max} and R_{min} are the maximal and minimal Fura 2 ratios in the presence and absence of Ca^{2+}, and F is the ratio of Fura 2 intensities at 380 nm in the presence and absence of Ca^{2+}.

RT-PCR ASSAYS

Total RNA from rat kidney (positive control), WT-, and AQP2-$RCCD_1$ cells were isolated using SV total RNA Isolation System (Promega, Madison, WI). Reverse transcription was performed on 2 μg of total RNA using M-MLV reverse transcriptase (Promega). RNAs were placed in 50 μl of "RT reaction buffer" containing: 1× M-MLV reverse transcriptase buffer, 0.5 μg oligo-dt primer and 10 U/μl RNAsin. The reaction was heated 3 min at 80°C and cooled to 45°C. PCR buffer (25 μl) containing: 1× M-MLV reverse transcriptase buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, and 400 U M-MLV, was added to half of the reaction. Control experiments, in the absence of the enzyme M-MLV, were performed on the remaining 25 μl. RT reaction was carried out for 1 h at 45°C and stopped by heating 2 min at 95°C. PCR experiments were performed with 5 μl of the RT reaction using 5 pmol of specific primers:

TRPV1 (sense: 5′ GAAGATCGGGGTCTTGGCCTA 3′, anti-sense: 5′ CTCACTGTAGCTGTCCACCCCCAAA 3′). Cycling parameters were: (1) 94°C 10 min, (2) 94°C 1 min, 60°C 1:30 min, 72°C 1 min, for 35 cycles, and (3) 72°C 10 min.

TRPV4 (sense: 5′ CCAACCTGTTTGAGGGAGAG 3′, anti-sense: 5′ TGGCTGCTTCTCTACGACCT 3′). Cycling parameters were: (1) 94°C 5 min, (2) 94°C 30 s, 56°C 45 s, 72°C 1 min 30 s, for 35 cycles, and (3) 72°C 10 min.

β-actin specific primers (sense: 5′ CGGAACCGCTCATTGCC 3′; anti-sense: 5′ ACCCACACTGTGCCCATCTA 3′). Cycling parameters were: (1) 94°C 5 min, (2) 94°C 30 s, 56°C 45 s, 72°C 1 min 30 s, for 35 cycles, and (3) 72°C 10 min.

WESTERN BLOTTING STUDIES

TRPV4 expression was examined by Western blot using the rabbit polyclonal anti-rat TRPV4 antibody, generated against a peptide corresponding to amino acids 853–871 of rat TRPV4 (ACC-034, Alomone). Confluent WT and AQP2-$RCCD_1$ cells were washed three times in cold PBS and were incubated for 30 min at 4°C in a cold lysis buffer containing 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1% Triton 100, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml anti-pain, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Cells were then collected with a rubber scraper and homogenized using a 21 gauge syringe needle. The homogenates were subject to SDS–PAGE 7.5% using the Tris/Tricine buffer system [Schägger and von Jagow, 1987] and transferred to nitrocellulose membranes (Mini Protean II, Bio-Rad). Blots were blocked with 5% nonfat dried skimmed milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween-20, pH 7.5) for 1 h and incubated with the anti-TRPV4 antibody (dilution 1:1,500) overnight at 4°C. The blots were then washed and incubated 1 h with a goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1:25,000 at 25°C; Sigma–Aldrich). Membranes were visualized using the chemiluminescence method (SuperSignal Substrate, Pierce) and captured on a Gbox (Syngene, Frederick, MD). The parity in protein loading in all blots was first verified by Ponceau S staining (Sigma–Aldrich).

IMMUNOFLUORESCENCE STUDIES

For TRPV4 staining, cells were fixed in 3% paraformaldehyde for 30 min and then permeabilized with 0.2% Triton X-100 at room temperature. Samples were blocked with 1% bovine serum albumin (BSA) and incubated with the primary antibody (TRPV4, 1/1,500) overnight at 4°C. Then the cells were washed and incubated with secondary antibody (CY3 conjugated goat anti rabbit IgG, Jackson Immuno) for 2 h at room temperature. Coverslips were mounted with Vectashield mounting medium. Images were captured and digitalized using a confocal Nikon C1 microscope.

In some experiments, colocalization of TRPV4 and the specific plasma membrane marker 488-conjugated wheat germ agglutinin (WGA; Molecular Probes) were analyzed. Cells were first fixed in paraformaldehyde and stained with 488-WGA, at 4°C for 30 min to label the surface glycoproteins of the plasma membrane. Then, cells

were permeabilized and treated as was above described with the anti-TRPV4 antibody, using a CY3-labeled secondary antibody.

For TRPV4/AQP2 colocalization experiments, cells were sequentially incubated with anti-AQP2 (Santa Cruz sc-9882, 1/50, overnight 4°C) and with anti-TRPV4 (1/1,500, overnight 4°C). Bovine anti-goat IgG-FITC and donkey anti-rabbit IgG-Cy5 were, respectively, used as second antibodies. Images were captured using confocal microscope Olympus Fluoview (FV1000).

SOLUTIONS AND CHEMICALS

Isosmotic solution (osmolality: 320 ± 4 mOsM) contained (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. Calcium-free solutions were made by adding EGTA (1 mM) and replacing $CaCl_2$ by $MgCl_2$.

Hyposmotic solutions (ΔOsM = 100 mOsM) were prepared by mannitol removal from the isosmotic solution, thus maintaining the ionic strength. A more diluted solution was obtained by direct dilution of the isosmotic solution with distilled water to reach a final osmolarity of 120 ± 3 mOsM (ΔOsM = 200 mOsM).

The osmolalities were routinely measured by a pressure vapor osmometer (Werked). All solutions were titrated to pH 7.40 using Tris (Sigma–Aldrich) and bubbled with atmospheric air.

In some experiments 10 μM of ruthenium red (RR) (Sigma–Aldrich), 1 or 10 μM 4α-phorbol-12,13-didecanoate (4α-PDD) (Sigma–Aldrich), 100 μM capsaicin, 2.5 μM colchicine, and 4 μM cytochalasin D were used. BCECF-AM (2 mM) and FURA 2 (1 mM) stock solutions were dissolved in dimethylsulfoxide (DMSO) and stored at −20°C until used.

STATISTICS

Values are reported as means ± SEM, and n is the number of cells evaluated from three to six different experiments. For all comparisons Student's *t*-test for unpaired data was applied and $P < 0.05$ was considered to be statistically significant.

RESULTS

RUTHENIUM RED INHIBITS CALCIUM ENTRY AND RVD IN AQP2-RCCD$_1$ CELLS

In order to investigate if a member of the TRPV family is involved in the increase in $[Ca^{2+}]_i$ necessary for rapid activation of RVD mechanisms in AQP2-RCCD$_1$ cells, we pretreated RCCD$_1$ cells with RR, a general blocker of TRPV-type channels. Our results show that 10 μM RR reduced the increase in calcium concentration ($\Delta[Ca^{2+}]_i^{max}$), induced by hypotonic exposure of AQP2-RCCD$_1$ cells, to a level similar to that observed in control WT-RCCD$_1$ cells (Fig. 1A). A higher hypotonic gradient (ΔOsM = 200 mOsM) further increases $[Ca^{2+}]_i$ levels, however it is blocked with RR to a value similar to that observed with ΔOsM = 100 mOsM + RR (Fig. 1B).

The effect of RR on the RCCD$_1$ cell volume response to hypotonic shock was also investigated. In this condition the percentage of RVD at 20 min (%RVD$_{20}$) was significantly reduced in AQP2-RCCD$_1$ cells but not in WT-RCCD$_1$ cells (Fig. 1C). The % RVD$_{20}$ in AQP2-RCCD$_1$ cells treated with RR was similar to the % RVD in control WT-RCCD$_1$ cells.

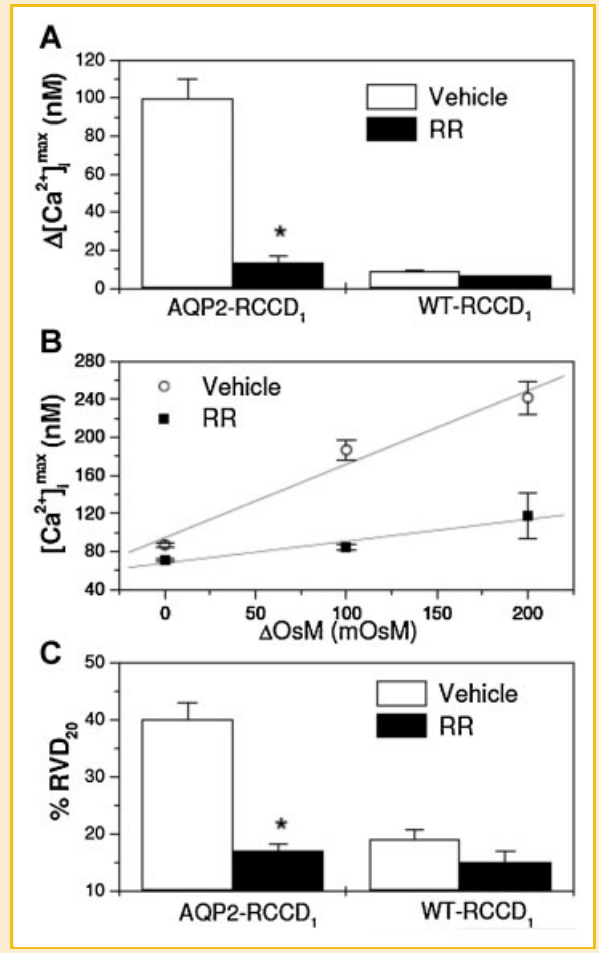

Fig. 1. Effects of ruthenium red (RR) on calcium entry and RVD in WT- and AQP2-RCCD$_1$ cells exposed to hypotonicity. A: $\Delta[Ca^{2+}]_i^{max}$ representing the maximal $[Ca^{2+}]_i$ increase after hypotonic shock, in WT- and AQP2-RCCD$_1$ cells, in the presence or absence of RR. Intracellular $[Ca^{2+}]_i$ was measured in Fura-2-loaded cells incubated with 10 μM RR or vehicle (DMSO) for 5 min in isotonic conditions and then exposed to a hypotonic shock (ΔOsM = 100 mOsM). B: Effects of varying external osmolarity on intracellular $[Ca^{2+}]_i$ levels, measured as indicated in (A), in AQP2-RCCD$_1$ cells exposed either to vehicle (DMSO) or 10 μM RR. C: Percentage of cell volume recovery at 20 min (% RVD$_{20}$). Cell volume changes were measured in BCECF-loaded cells incubated with 10 μM RR or vehicle (DMSO) for 5 min in isotonic conditions and then exposed to a hypotonic shock (ΔOsM = 100 mOsM). Values are mean ± SEM for 30–100 cells from 3 to 5 experiments, $^*P < 0.001$.

These results are consistent with our hypothesis that a TRPV channel could mediate Ca^{2+} entry during swelling of AQP2-RCCD$_1$ cells and that it may have a key role in generating the RVD response.

TRPV4 IS EXPRESSED AND IT IS FUNCTIONAL IN AQP2- AND WT-RCCD$_1$ CELLS

Of the six mammalian TRPV channels, the TRPV1, TRPV2, and TRPV4 have been shown to have a role in transduction of osmotic and mechanical stimuli [Liedtke, 2006]. Of these, only TRPV1 and TRPV4 have been described in kidney cells [Tian et al., 2004; Taniguchi et al., 2007; Wu et al., 2007; Feng et al., 2008]. So the next

step was to investigate the function and expression of these channels in $RCCD_1$ cells.

Figure 2A shows that in the absence of any osmotic gradient capsaicin, a specific activator of TRPV1, is not able to induce an important increase in calcium neither in AQP2- nor in WT-$RCCD_1$ cells. In contrast 4α-PDD, a selective activator of TRPV4, increased calcium concentration in both AQP2- and WT-$RCCD_1$ cells and these increases were significantly inhibited by RR.

To examine whether TRPV1 and TRPV4 mRNA are expressed in WT- and AQP2-$RCCD_1$ cells, we performed RT-PCR assays. Rat kidney mRNA was used as positive control. β-Actin primers were employed as control of the mRNA (data not shown). Figure 2B shows that while the kidney expresses mRNA of TRPV1 it is absent in AQP2- and WT-$RCCD_1$ cells. This result is consistent with the lack of response to capsaicin. In contrast, in the experiments performed using primers for TRPV4 a band of expected size (287 bp) was obtained in the kidney sample and in both cell types. We did not find expression of TRPV2, TRPV3, TRPV5, and TRPV6 by RT-PCR assays in AQP2 and WT-$RCCD_1$ cells (Supplementary Fig. 1).

In addition immunoblots of kidney and cell lysates revealed the presence of TRPV4 protein in all of the samples (Fig. 2C). The specificity of the affinity-purified antibody to the 857–871 amino acids of rat TRPV4 used in our study was confirmed by blocking of the TRPV4 Western blot signal with antigen preincubation.

All these results indicate that TRPV4 is expressed and that it is also functional in both cell lines, however, a large activation of the channel by hypotonicity only occurs in cells that express AQP2.

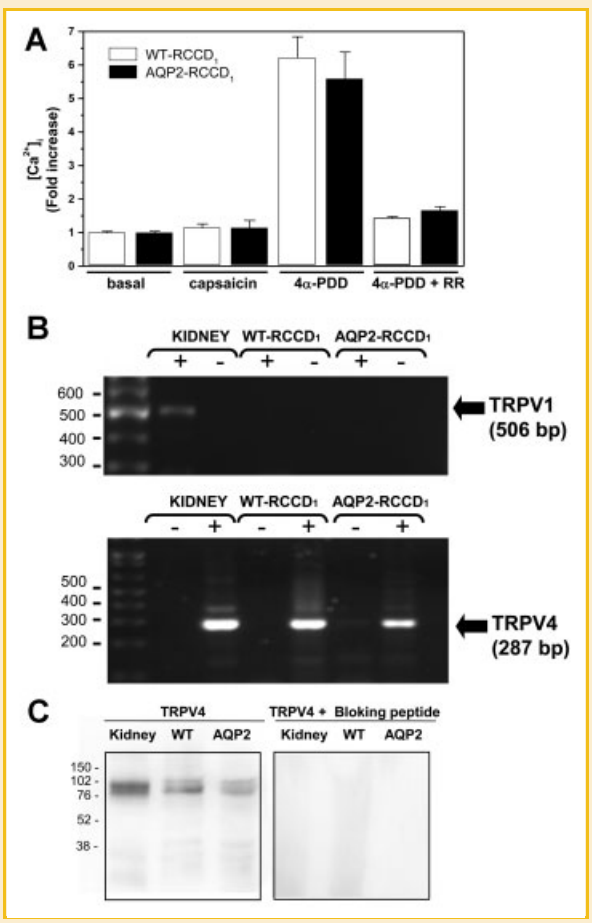

Fig. 2. Functional and molecular expression of TRPV4 in WT- and AQP2-$RCCD_1$ cells. A: Effects of the specific activators of TRPV1 (capsaicin, 100 μM) and of TRPV4 (4α-PDD, 1 μM) either alone or in the presence of 10 μM RR on calcium entry in isotonic conditions. Values are expressed as fold increase respect to basal value and represent the mean ± SEM for 40–100 cells from 3 to 5 experiments. B: Representative RT-PCR experiments performed using specific primers for rat TRPV1 and TRPV4 in mRNAs obtained from rat kidney (positive control), WT- and AQP2-$RCCD_1$ cells. Assays were carried out in the presence (+) or absence (−) of MMLV enzyme. C: Representative Western blot using a rat TRPV4 antibody (dilution 1:1,500) in lysates obtained from kidney, WT-, and AQP2-$RCCD_1$ cells. At least three independent experiments were performed. The reactivity was blocked by preincubation of the antibody with the blocking peptide.

ACTIVATION OF TRPV4 WITH 4α-PDD ELICITS RVD IN WT-$RCCD_1$ CELLS

We then evaluated whether the rapid activation of RVD response absent in WT-$RCCD_1$ cells could be evoked after specific activation of TRPV4. To do this, cells were exposed to hypotonicity in the presence of 4α-PDD (Fig. 3). It can be observed that in WT-$RCCD_1$ cells the combination of both stimuli resulted in an increase in the % RVD_{20} (Fig. 3A) and in the amplitude of the Ca^{2+} response as compared with the vehicle (Fig. 3B). Furthermore, TRPV4 specific activation by 4α-PDD in isotonic conditions induced a significant decrease in cell volume in both cells lines (Fig. 3C). These results are in line with the hypothesis that activation of TRPV4 could result in a rapid triggering of RVD mechanisms.

CYTOSKELETON DEPOLYMERIZATION DECREASED THE AMPLITUDE OF HYPOTONIC-ACTIVATED CALCIUM TRANSIENTS IN AQP2-$RCCD_1$ CELLS

A functional interaction between TRPV4 and cytoskeleton in sensing hypotonicity and the onset of RVD have been previously described in HaCaT keratinocytes and CHO cells. [Becker et al., 2009]. Therefore, we evaluated whether the increase in Ca^{2+} levels and the consequent RVD response only observed in cells expressing AQP2 were dependent of cytoskeleton integrity. Therefore, AQP2-$RCCD_1$ cells were exposed to two cytoskeleton depolymerization agents (colchicine and cytochalasin D). Figure 4A and inset show that cytochalasin D-treatment decreased the amplitude of hypotonic-activated calcium transients while disruption of tubulin by colchicine avoids this increase (Fig. 4B, inset), indicating that actin filaments and microtubules modulate hypotonic-activated calcium transients. In parallel, treatment with cytochalasin D or colchicine during the hyposmotic shock induced the expected swelling but show a significant RVD inhibition (Fig. 4C). All these data demonstrate that the integrated arrangement of cytoskeleton is essential for activation of Ca^{2+} entry and subsequent volume regulation in cells expressing AQP2.

UNDER HYPOTONIC SHOCK TRPV4 IS TRANSLOCATED TO THE CELL MEMBRANE IN AQP2 BUT NOT IN WT-$RCCD_1$ CELLS

Participation of microtubules in the swelling activation of the TRPV4 by hypotonic stimuli suggests the involvement of a traffic process in AQP2-$RCCD_1$ cells. Therefore, the next step was to study

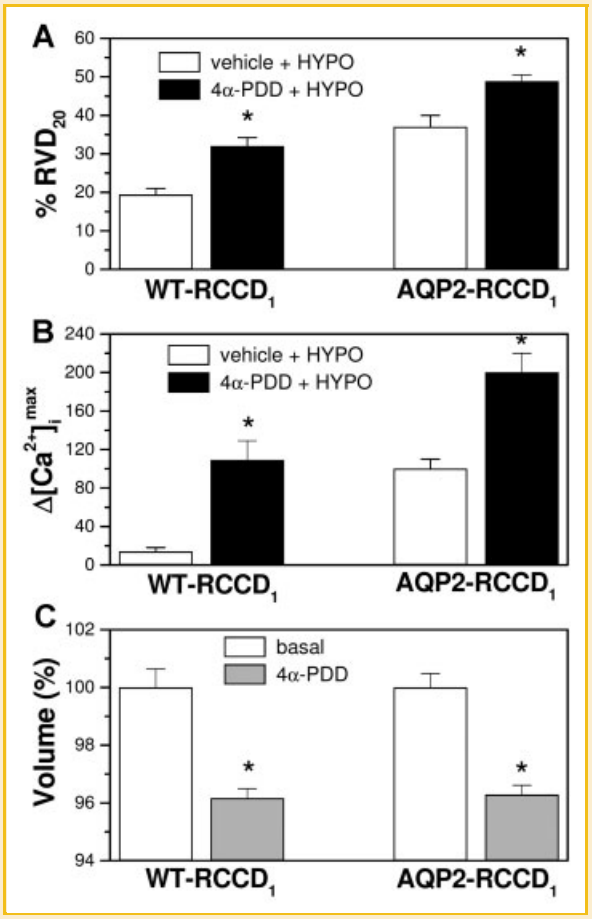

Fig. 3. Effects of 4α-PDD on RVD and calcium entry in WT- and AQP2-$RCCD_1$ cells. A,B: BCECF- or Fura-2-loaded cells were incubated with the TRPV4 activator 4α-PDD (1 μM) or vehicle (DMSO) and then exposed to a hypotonic shock (ΔOsM = 100 mOsM). Percentage of cell volume recovery at 20 min (% RVD_{20}) (A) and $\Delta[Ca^{2+}]_i^{max}$ representing the maximal $[Ca^{2+}]_i$ increase after hypotonic shock (B). C: Cell volume measured before (basal) and after 20 min of treatment with 4α-PDD (1 μM) in isotonic conditions (expressed as %). Values are mean ± SEM for 30–100 cells from 3 to 5 experiments, $^*P < 0.01$.

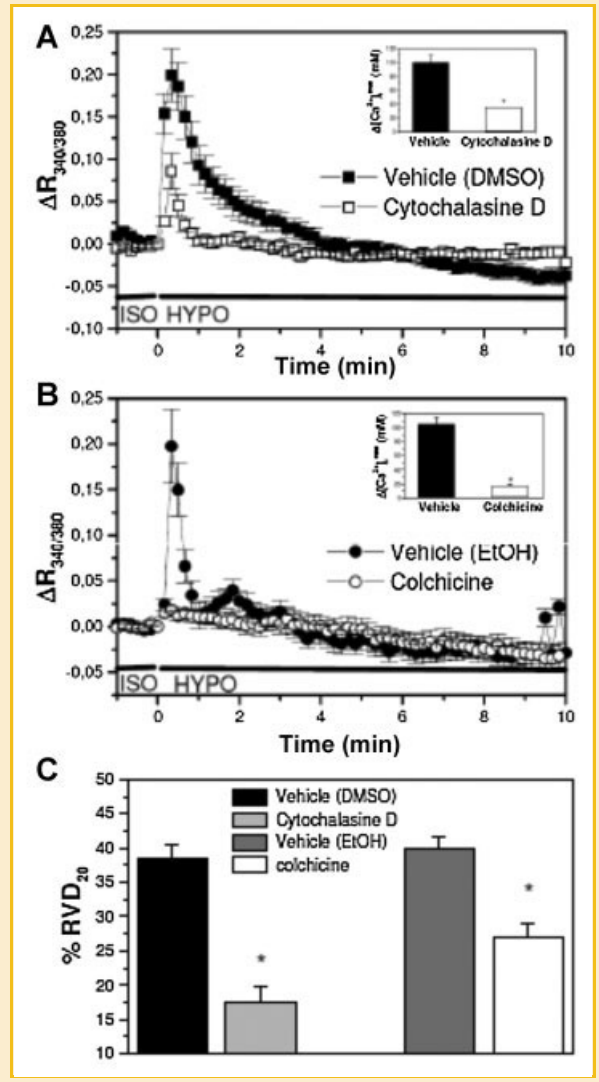

Fig. 4. Effects of cytochalasin D and colchicine on calcium entry and RVD in AQP2-$RCCD_1$ cells exposed to hypotonicity. Dynamics of the fluorescence ratio in AQP2-Fura-2-loaded cells ($\Delta R_{340/380}$) pre-incubated with 4 μM cytochalasin D or vehicle (DMSO) for 60 min (A) and with 2.5 μM colchicine or vehicle (EtOH) for 30 min in isotonic conditions (B), after the treatment cells were exposed to a hypotonic shock (ΔOsM = 100 mOsM). Insets show $\%[Ca^{2+}]_i^{max}$ in each condition above described. C: Percentage of cell volume recovery at 20 min (% RVD_{20}) in the conditions described in (A) and (B). Values are mean ± SEM for 30–100 cells from 3 to 5 experiments, $^*P < 0.01$ vehicle versus treated cells.

the localization of TRPV4 as well as investigating if hypotonicity could change the location of the protein. Thus we performed immunofluorescence studies with affinity purified TRPV4 antibodies in AQP2 and WT-$RCCD_1$ cells both in isosmotic and hyposmotic conditions (Fig. 5A). Confocal microscopy images illustrate that both cell lines, in isotonic conditions, show a preferentially intracellular distribution with a much smaller percentage mark in the cell membrane (Fig. 5a,c). On the other hand, hypotonic stimulation treatment (ΔOsM = 100 mOsM) revealed that while in WT-$RCCD_1$ cells no changes in distribution were observed, in AQP2-$RCCD_1$ cells the pattern was quite different, showing that TRPV4 was enriched in the membrane of cells expressing AQP2 (Fig. 5b,d). A similar pattern was observed when cells were treated with a higher gradient (ΔOsM = 200 mOsM) (data not show). The hypotonic-induced TRPV4 trafficking was inhibited by pretreatment of AQP2-$RCCD_1$ cells with 2.5 μM colchicine, for 30 min (Fig. 5e,f).

To gain further insight in the subcellular localization of TRPV4, immunofluorescence confocal microscopy analysis was performed on $RCCD_1$ cells co-stained with the selective fluorescent marker of glycosylated surface-expressed proteins WGA-Alexa Fluor 488 [Wright, 1984]. Figure 5B shows AQP2-$RCCD_1$ cells labeled with anti-TRPV4 (red) and WGA-Alexa Fluor 488 (green) under Iso (top panel) and hypotonic (lower panel) conditions. Merge of single plane confocal images demonstrates a higher overlap of TRPV4 staining with the plasma membrane in hypotonic as compared with isotonic conditions.

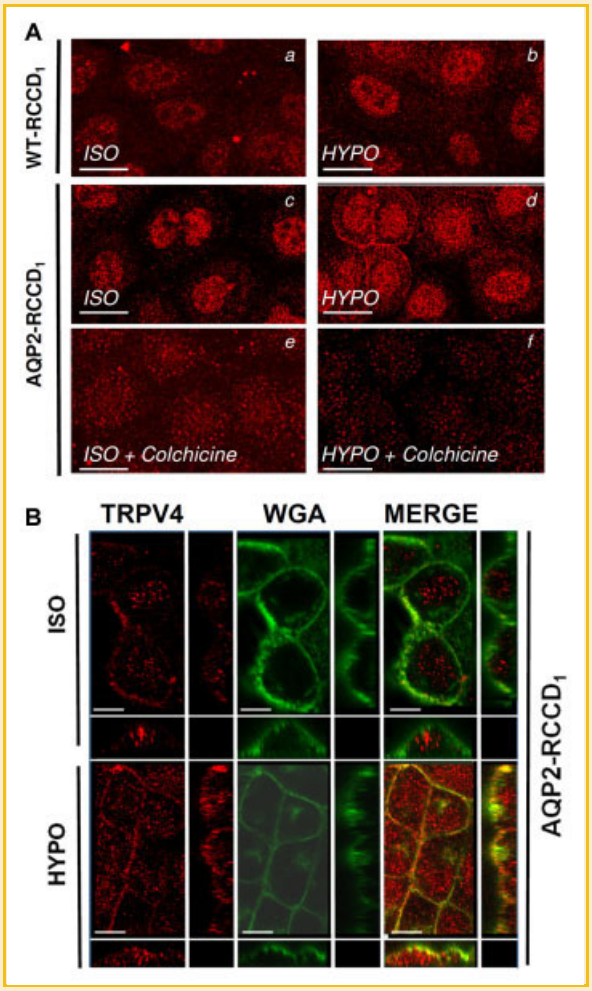

Fig. 5. TRPV4 localization in WT- and AQP2-$RCCD_1$ cells exposed to hypotonicity. A: Immunofluorescence experiments performed using a primary anti-TRPV4 antibody and the secondary Cy3-antibody. Confocal images of WT- and AQP2-$RCCD_1$ cells maintained in isotonic (a, c, and e) conditions and then exposed for 5 min to hypotonicity ($\Delta OsM = 100$ mOsM) (b, d, and f). AQP2-$RCCD_1$ cells were also pre-incubated with 2.5 μM colchicine for 30 min in isotonic conditions (e) and after a hypotonic shock (f). Scale bars: 10 μm. B: TRPV4 colocalization with WGA in AQP2-$RCCD_1$ cells exposed to hypotonic conditions. Immunofluorescence experiments performed using a primary anti-TRPV4 antibody and the secondary Cy3-antibody (red), 488-conjugated wheat germ agglutinin (WGA; Molecular Probes) (green) and merged staining in AQP2-$RCCD_1$ cells. Confocal images show a single xy plane and the cross-section in xz and yz planes of cells maintained in isotonic conditions (A) or exposed for 5 min to hypotonicity ($\Delta OsM = 100$ mOsM) (B). Scale bars: 10 μm.

TRPV4 AND AQP2 ARE FUNCTIONALLY INTERACTING

All results presented above indicate that AQP2 cells respond to hypotonic shock by increasing TRPV4 expression in their cell membrane. In order to test if a physical interaction could be possible between AQP2 and TRPV4 we performed colocalization experiments. Cells were double stained sequentially with anti-AQP2 and anti-TRPV4 antibody. Figure 6A shows that although a hypotonic shock increases both AQP2 and TRPV4 expression at the membrane domains, an important colocalization does not seem to appear. Hence the interaction between AQP2 and TRPV4 could be a functional relationship. We have previously demonstrated in AQP2-$RCCD_1$ cells that treatment with $HgCl_2$ reduced the osmotic water permeability [Ford et al., 2005], so we now evaluated whether the functional inhibition of AQP2 affects translocation/activation of TRPV4. Figure 6B shows that preincubation of AQP2-$RCCD_1$ cells with $HgCl_2$ inhibits the hypotonic induced traffic of TRPV4 to the cell membrane. In parallel, in this condition we found that intracellular calcium increase after hypotonic shock was significantly reduced ($\Delta[Ca^{2+}]_i^{max}$ (nM), control: 155 ± 15 vs. $HgCl_2$: 7.18 ± 1.15; $P < 0.001$, $n = 30$ cells from three experiments). Altogether these results let us propose a functional interaction between AQP2 and TRPV4.

DISCUSSION

TRPV4 has been shown to be implicated in the process of volume regulation in many cell types; however, its role in RVD has not been previously shown in renal cells [Arniges et al., 2004; Becker et al., 2005; Liu et al., 2006; Pan et al., 2008]. We here demonstrated, in AQP2-$RCCD_1$ cells that the Ca^{2+} entry in response to cell swelling and the subsequent RVD were sensitive to RR. Although not specific it is a compound fully used as TRPV4 inhibitor [Güler et al., 2002; Becker et al., 2005; Jian et al., 2008; Pan et al., 2008]. Here we have shown that $RCCD_1$ cells do not respond to the activator of TRPV1, capsaicin, and do not express TRPV1, TRPV2, TRPV3, TRPV5, and TRPV6. In contrast $RCCD_1$ cells respond to the specific activator of TRPV4, 4α-PDD, and express endogenous TRPV4 channels, in agreement with previous reports performed in mouse CCD cells [Wu et al., 2007]. Interestingly, it has been reported that the endogenous TRPV4 protein expressed in mouse CCD cells is sensitive to mechanical stimuli including fluid flow/shear stress as well as hypotonic cell swelling suggesting that the TRPV4 channel would appear to function as a molecular sensor of flow and osmolality (hypotonicity) or as a component of a renal epithelial sensing complex [Taniguchi et al., 2007; Wu et al., 2007]. However, the authors did not investigate the role of TRPV4 during RVD. Despite the here observed endogenous expression of TRPV4 channels in both cell lines, only cells expressing AQP2 are able to increase calcium under a hypotonic shock. On the contrary, in the absence of any osmotic gradient, WT- and AQP2-$RCCD_1$ cells respond in the same way to the specific activator 4-αPDD showing that the lack of calcium increase observed in WT-$RCCD_1$ cells under hypotonicity is not due to an expression of a nonfunctional TRPV4. The different way of TRPV4 activation described in this work is in accordance with previous observations reporting that cell swelling and chemical agonists use distinct pathways for the activation of TRPV4 [Arniges et al., 2004; Vriens et al., 2004]. Interestingly, we showed that WT-$RCCD_1$ cells are able to trigger the RVD response if TRPV4 is previously activated with 4α-PDD. Therefore, the presence of a functional AQP2 is necessary to trigger the pathway involved in hypotonic TRPV4 activation and then elicit a rapid RVD response.

Conflicting data have emerged concerning the molecular mechanism through which hypotonicity influences TRPV4 function. Xu et al. [2003] noted that TRPV4 undergoes tyrosine phosphorylation, in response to hypotonic stress, by SRC-family cytoplasmic

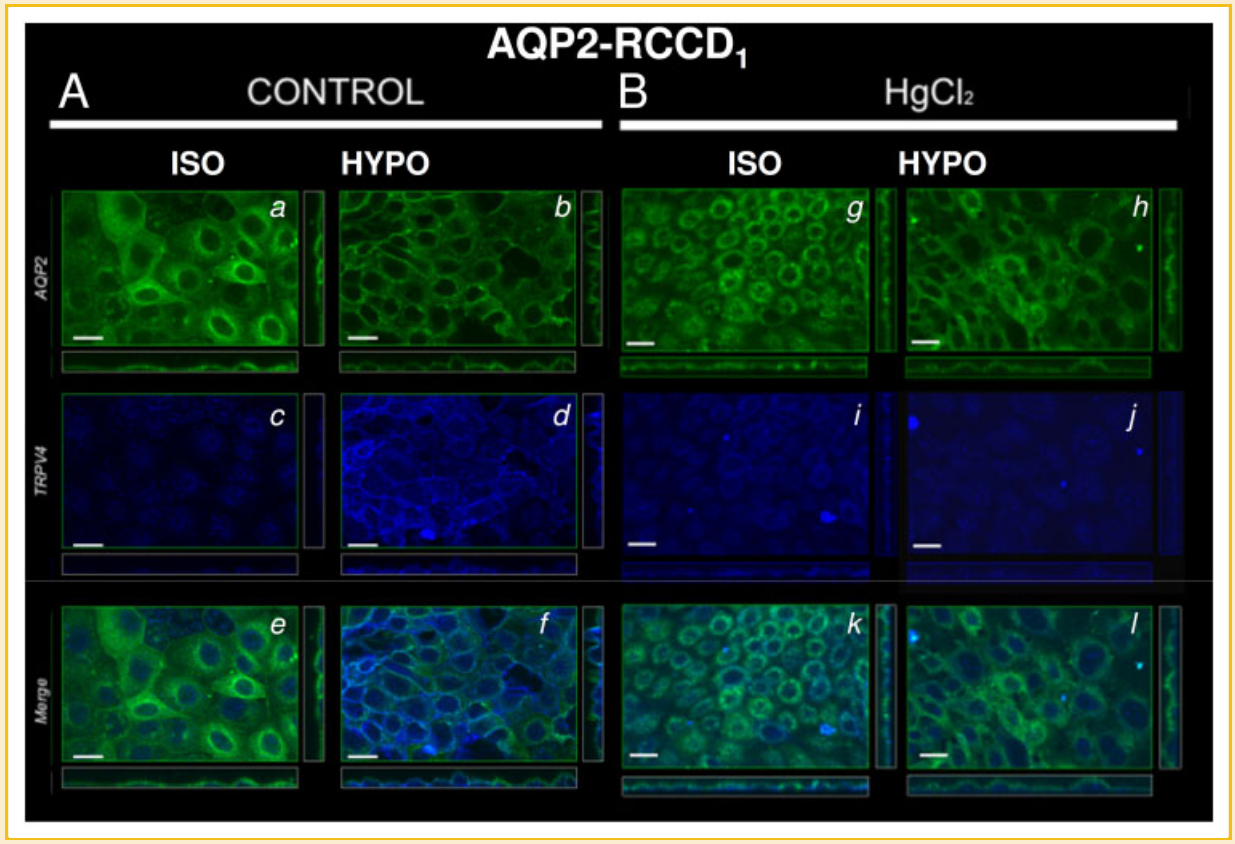

Fig. 6. Effects of AQP2 inhibition on TRPV4 localization in AQP2-RCCD$_1$ cells exposed to hypotonicity. Confocal microscopy analysis of xy, xz, and yz illustrating AQP2, TRPV4 and merged staining in AQP2 cells exposed either to isotonic (a, c, e, g, i, k) or hypotonic solutions (b, d, f, h, j, l) (ΔOsM = 200 mOsM) in the absence (panel A: a, b, c, d, e, f) or presence of 300 μM $HgCl_2$ (panel B: g, h, i, j, k, l). Co-immunofluorescence experiments were performed using a primary anti-TRPV4 antibody and the secondary Cy-5 (blue) and the polyclonal anti-AQP2 antibody and the secondary FITC (green). Scale bars: 10 μm.

tyrosine kinases. In contrast to these findings, Vriens et al. [2004] propose that phospholipase A_2 (PLA_2) is essential for TRPV4 activation by hypotonic stress but not by the lipid agonist, 4α-PDD. It has also been suggested that the mechanisms involved in activation of TRPV4 by swelling should be a channel translocation to the plasma membrane [Cuajungco et al., 2006; Pedersen and Nilius, 2007]. Our immunofluorescence experiments revealed that in the absence of any osmotic gradient TRPV4 distributes similarly in WT- and AQP2-RCCD$_1$, located mostly in the cytoplasm, probably in endoplasmic reticulum, in Golgi or in exocytic vesicles, and to a lesser extent, in the plasma membrane. In contrast, in the presence of a hypotonic medium TRPV4 accumulates in the plasma membrane only in cells expressing functional AQP2. The translocation of TRPV4 to the plasma membrane of RCCD$_1$ cells was confirmed by colocalization of TRPV4 with the fluorescent membrane marker WGA. In addition, our results indicate that cytoskeleton integrity is required for TRPV4 translocation/activation and for the consequent RVD in cells expressing AQP2. This is consistent with studies in various cell types that reported that intact actin cytoskeleton is required for calcium entry and RVD after a swelling [Becker et al., 2005; Ebner et al., 2005; Liu et al., 2006].

All results here presented let us suggest a putative interaction between AQP2 and TRPV4. Under hypotonic stimuli TRPV4 could be physically associated with AQP2 in the apical membrane as previously shown for a member of the TRP family (TRPC3) and AQP2 in CCD stimulated with AVP [Goel et al., 2007]. Nevertheless, our results do not show an evident colocalization of AQP2 and TRPV4 in renal cells subjected to hypotonic medium, except at some points of the membrane. However the reduction of AQP2 function with $HgCl_2$ avoids not only TRPV4 translocation but also calcium increase, suggesting a functional, more than a physical, interaction between both molecules.

Although in the present paper we have not investigated the mechanism linking the presence of AQP2 and the translocation of the TRPV4, some evidences exist that allow us to suggest a putative role of the PLA2-epoxieicosatrienoic acids (EET) pathway. (1) It has recently been reported, in endothelial cells that activation of the TRPV4 channel by flow requires CYP epoxygenase-derived EETs and the translocation of the channel to the cell membrane [Loot et al., 2008], (2) the activation of PLA2 and the subsequent formation of AA and EETs is a process that is considered to be part of the mechano-osmosensing in different cells, since the PLA2 can be triggered by the osmotic swelling or by the mechanical stimuli [Lehtonen and Kinnunen, 1995], and (3) an interaction between AQP1 and PLA2 has been previously reported in the regulation of secretory vesicle swelling in exocrine pancreas [Abu-Hamdah et al.,

2004]. We have preliminary data showing that while WT-RCCD$_1$ cells pretreated with 5.6-EET did not modify intracellular calcium concentration in isotonic conditions, calcium levels are significantly increased under a hypotonic shock [Galizia et al., unpublished results]. This result opens the possibility to study whether the presence of functional AQP2 may be important to stimulate some plasma membrane PLA with the consequent activation of the AA and EETs cascade leading to TRPV4 translocation/activation.

In summary, our data provide evidence, for the first time, of a novel association between the water channel AQP2 and the calcium permeable nonselective cation channel TRPV4, critical for calcium entry during hypotonicity and for the subsequent RVD response in CCD cells, suggesting that both proteins are assembled in a signaling complex that responds to anisosmotic conditions.

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