

Hypotonicity Induces Aquaporin-2 Internalization and Cytosol-to-Membrane Translocation of ICln in Renal Cells

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Kidney collecting-duct cells swell in response to changes in medulla osmolality caused by the transition from antidiuresis to diuresis. Regulatory volume decrease (RVD) mechanisms must be activated to face this hypotonic stress. In Aquaporin-2 (AQP2)-expressing renal CD8 cells, hypotonicity decreased cell surface expression of AQP2 and increased the amount of AQP2 localized intracellularly, whereas the total amount of AQP2 phosphorylated at ser-256 decreased. Analysis of cAMP dynamics using fluorescence resonance energy transfer (FRET) showed that hypotonicity causes a reduction of cAMP, consistent with a decrease in phospho-AQP2. Moreover, hypotonicity caused a profound actin reorganization, associated with the loss of stress fibers and formation of F-actin patches

(microspikes) at the cell border. Those changes were regulated by the monomeric GTPase Cdc42. Interestingly, expression of the dominant-negative Cdc42 (N17-Cdc42) prevented the hypotonicity-induced microspike formation and the generation of Cl^- currents. Hypotonicity also caused the relocation from the cytosol to the plasma membrane and increase in interaction with actin of ICln (nucleotide-sensitive chloride current protein), which is essential for the generation of ion currents activated during RVD. Together, the profound actin remodeling, internalization of AQP2 and translocation of ICln to the plasma membrane during hypotonicity may contribute to RVD after cell swelling in renal medulla. (*Endocrinology* 148: 1118–1130, 2007)

ONE FUNDAMENTAL PROPERTY of mammalian cells is their ability to regulate their volume within a wide range of the external osmolality. When cells are exposed to hypotonic extracellular fluid, they swell because of the osmotic water influx (1, 2). After the swelling, cells can return to their original volume through a complex mechanism called regulatory volume decrease (RVD). This process is based on the efflux of ions (usually Cl^- and K^+) and osmolytes followed by water efflux along the osmotic gradient (2–4). In contrast, in response to an increase in extracellular osmolality, cells shrink and eventually regain their original volume in a complex mechanism known as regulatory volume increase (RVI). The latter is usually effected by an influx of sodium and chloride. The uptake of NaCl with osmotically obliged water leads to an increase in cell volume to counteract cell shrinkage (2, 5, 6). Kidney cells are often exposed to great osmotic challenges. As a consequence, kidney tubule cells have a strong requirement for cell volume regulatory

mechanisms because they have to face severe perturbations of their cell volume.

This is particularly true for the cells in the kidney medulla. In the presence of the antidiuretic hormone arginine vasopressin (AVP), there is an osmotic gradient in the kidney medulla between the interstitium and tubular lumen (7–9). This gradient is the driving force for renal water reabsorption. AVP binds to the V2 receptor located in the basolateral membrane of collecting duct principal cells and triggers an intracellular signaling cascade, increasing cAMP levels with consequent activation of protein kinase A (PKA) (10). PKA-dependent phosphorylation of the water channel aquaporin 2 (AQP-2) is a key signal regulating redistribution of intracellular AQP2-bearing vesicles to the apical membrane, which becomes water permeable (8, 9). Because of the presence of the corticomedullary gradient, water moves through the apical membrane into the cells and leaves the basolateral membrane via AQP3/AQP4, thus increasing urine concentration. This is a short-term effect (7, 11–13). In fact, interaction of the V2 receptor with AVP leads to activation of the receptor, followed by termination of the response by down-regulation, accomplished by a variety of signals including receptor internalization, uncoupling from the G protein, and degradation of cAMP by cytosolic phosphodiesterase (14). This causes the transition from an antidiuretic to a diuretic condition, which is associated with a decrease in medulla osmolality, causing cell swelling. It has been shown that after the transition from an antidiuretic to a diuretic condition by

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Abbreviations: anti-AQP2, AQP2 antibodies; antiphospho-AQP2, antiphosphorylated AQP2 antibodies; AQP, Aquaporin; AVP, arginine vasopressin; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; ICln, nucleotide-sensitive chloride current; LIMK, LIM kinase; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; RVD, regulatory volume decrease; YFP, yellow fluorescent protein.

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inhibition of NaCl transport systems in rat kidneys with furosemide, the nucleotide-sensitive chloride current (ICln) protein is rapidly translocated to the membrane of the distal tubule cells (15). Under these diuretic conditions, the distal tubule cells are supposed to swell, therefore activating mechanisms that allow these cells to counterbalance cell swelling, which would otherwise impair cell and ultimately organ function. ICln is a multifunctional protein (16) that is essential for the generation of ion currents activated during RVD after cell swelling (17). The heterologue expression of ICln leads to a current similar to the endogenous RVD current observed in most mammalian cells, qualifying ICln as one of the molecular entities for the ion channel activated during RVD (5, 18). To allow distal tubule cells to effectively regulate their volume after swelling, besides providing pathways for the ion exit, it would also be advantageous to limit water entry from the apical side. Therefore, the aim of this work was to investigate how principal cells in the inner medulla expressing AQP water channels can react to changes during hypotonicity. To this end, we used AQP2-expressing renal CD8 cells endogenously expressing the ICln protein. In particular, we evaluated the major alterations induced in CD8 cells during a short-term hypotonic shock and investigated whether a cross talk between ICln and AQP2 may play an osmoregulatory role in mammalian kidney collecting duct, a conditionally water-impermeant tubule segment characterized by a substantial transepithelial osmotic gradient.

Materials and Methods

Antibodies

AQP2 antibodies (anti-AQP2) were raised against a synthetic peptide corresponding to the 15 COOH-terminal amino acids of human AQP2 plus an N-terminal cysteine used for conjugation (Cys-Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Thr-Lys-Ala). The peptide was conjugated to BSA by a cysteine sulfhydryl linkage and used for immunizing rabbits. Antisera were affinity-purified by passage over a SulfoLink kit (Pierce, Rockford, IL) column to which the peptide had been attached covalently. The purified antihuman AQP2 antibodies were eluted at pH 2.5 and quickly titrated to pH 7.5.

Antiphosphorylated AQP2 antibodies (antiphospho-AQP2) were prepared according to the procedure published by Nishimoto *et al.* (19) with some modifications. Briefly, a peptide corresponding to amino acids 253–262 of human AQP2 was synthesized with the addition of a cysteine residue at the carboxyl terminus and a glycine residue at the amino terminus [Gly-Arg-Arg-Gln-Ser(PO₃H₂)-Val-Glu-His-Leu-Ser-Pro-Cys-NH₂]. In the peptide sequence, Ser256 in the PKA consensus sequence (Arg-Arg-Gln-Ser) was phosphorylated. Downstream from the PKA consensus sequence, amino acids 259 (Leu) and 260 (His) were switched to reduce the antigenicity of this region. The peptide was conjugated to ovalbumin via the terminal cysteine and used to immunize rabbits. To remove IgG recognizing nonphosphorylated AQP2, the whole antiserum was applied three times to a column to which a non-phosphorylated peptide, reproducing amino acids 257–271 of human AQP2 with a cysteine residue at the amino terminus (Cys-Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Thr-Lys-Ala), was conjugated. The anti-AQP2 antibody depleted serum was then applied to a second column to which the phosphorylated AQP2 peptide for affinity purification was conjugated. The specificity of these affinity-purified antibodies was tested by Western blotting and by ELISA. For ELISA, increasing concentrations (100, 200, 300, 400, 500, 1000 pg per 50 μ l) of synthetic peptide reproducing the nonphosphorylated and the phosphorylated C-terminal region of the human AQP2 were adsorbed onto 96-well plates for 16 h at 4 C. Wells were then incubated with a blocking solution PBS containing 3% BSA at room temperature for 1 h. The affinity purified antiphospho-AQP2 (0.3 μ g/ μ l) was diluted in blocking solution (final antibody dilution 1:300) and added to each well for 2 h at 37 C.

Wells were then washed with washing buffer (PBS-0.1% Tween 20) and incubated with antirabbit IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO) for 1 h at 37 C. After five washings with washing buffer, 50 μ l of the substrate solution [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma] were added in each and incubated for 30 min in the dark. Absorbance was measured with a microplate reader at 405 nm (Bio-Rad 550; Bio-Rad Laboratories, Hercules, CA).

The phospho-AQP2 antibodies recognized the phospho-AQP2 peptide but not the non phosphorylated AQP2 peptide.

Cdc42, LIMK1, and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas cofilin and antiphospho-cofilin were from Cell Signaling (Beverly, MA). The ICln antibody was raised against a synthetic peptide reproducing the 24 C-terminal amino acids of the ICln protein and affinity purified (20).

Plasmids

RII-CFP and C-YFP have been previously described (21) and were kindly provided by M. Zaccolo (Venetian Institute of Molecular Medicine, Padova, Italy).

The N17-Cdc42 dominant-negative mutant (tagged with a Myc epitope at the N terminus) was subcloned from the pEXV vector into the EcoRI site of the pIRES2-EGFP plasmid (Clontech Laboratories, Inc., Palo Alto, CA). The cloned plasmid was termed pIRES2-EGFP-cdc42^{-/-} and the pIRES2-EGFP plasmid was used as the control.

Solutions

Cells were perfused with a phosphate buffer (pH 7.4, 310 mOsm) containing (in millimoles) 137 NaCl, 1 CaCl₂, 0.5 MgCl₂, and 2.7 KCl. The hypotonic solution had the same composition but with a reduced NaCl concentration of 45.6 mM. For the whole-cell patch-clamp experiments, the hypertonic bath solution contained (millimoles): 125 NaCl, 2.5 MgCl₂, 2.5 CaCl₂, 100 mannitol, and 10 HEPES/NaOH (pH 7.4). The hypotonic bath solution contained (millimoles): 125 NaCl, 2.5 MgCl₂, 2.5 CaCl₂, and 10 HEPES/NaOH (pH 7.4). The pipette solution contained (millimoles) 125 CsCl, 5 MgCl₂, 50 raffinose, 11 EGTA, 2 MgATP, and 10 HEPES/CsOH (pH 7.2).

Cell fractionation

For cell fractionation, confluent monolayers were either left untreated, in isotonic conditions, or incubated with the hypotonic solution for 10 min at 37 C. Cells were then rapidly scraped off the tissue culture plates and homogenized by three repeated freeze-thawing cycles (liquid nitrogen/37 C) and subsequently lysed in a buffer containing (in millimoles) 150 NaCl, 0.5 EDTA (EDTA), 0.5 MgCl₂, 10 Tris (pH 7.4), and 1% (vol/vol) Triton X-114, 1 phenylmethylsulfonyl fluoride (PMSF), 2 μ g/ml leupeptin, and 2 μ g/ml pepstatin A. Samples were left on ice for 5 min and then centrifuged at 14,000 $\times$ g for 15 min at 4 C to separate detergent-solubilized cell material (cytosol) and detergent-insoluble material (particulate). The insoluble pellet was completely solubilized in PBS containing 1% Nonidet-P40 and 0.5% Triton X-100 and 1 mM PMSF, 2 μ g/ml leupeptin, and 2 μ g/ml pepstatin A.

Alternatively, plasma membrane fractions were obtained using Qproteome plasma membrane kit according to the manufacturer's instructions (QIAGEN S.p.A., Milano, Italy).

To evaluate the total amount of AQP2 and phospho-AQP2, confluent monolayers, from CD8 cells, were either left untreated, in isotonic conditions, or incubated with the hypotonic solution for 10 min at 37 C. Cells were then rapidly scraped and lysated with a buffer containing (in millimoles) 50 Tris-HCl, 110 NaCl, 1 PMSF, 0.5% Triton X-100, and 0.5% Nonidet P-40.

To detect the amount of phospho-AQP2 from kidney tubule, tubule suspension was obtained from male Wistar rats. Rats were maintained at 25 C and had access to standard laboratory chow and water *ad libitum*. All procedures were in accordance with the guiding principles in the care and use of laboratory animals of Bari University. Rats were killed and kidneys were quickly excised and tubule suspension was obtained as previously described (22, 23).

The specificity of the antiphospho-AQP2 antibodies was also evaluated using a cell lysate from the RC.SV3 rabbit collecting duct cell line stably transfected with S256A AQP2 (24).

Analysis of association with actin cytoskeleton

The interaction of ICln with actin cytoskeleton was measured by its solubility in Triton X-100 as previously described (25). Proteins were resolved in 13% polyacrylamide gels and then transferred onto immobilon-P (Millipore, Bedford, MA) by standard procedures.

Coimmunoprecipitation

CD8 cells were washed with PBS and homogenized with a glass Teflon homogenizer in lysis buffer containing (in millimoles) 20 Tris-HCl, 1% Igepal, 1 EDTA, 1 EGTA, 1 dithiothreitol, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1.5 MgCl_2 , 0.15 M NaCl (pH 8.0). The homogenates were centrifuged at $17,000 \times g$, 30 min at 4 C and the supernatants were incubated overnight with ICln antibodies (10 μl). Protein A-conjugated agarose beads (Sigma) were added (30 μl) and incubated for 2 h. The beads were washed three times with 1 ml of lysis buffer. Bound proteins were eluted with Laemmli sample buffer without dithiothreitol, heated for 5 min at 95 C, and subjected to Western blot analysis using actin antibodies.

Fluorescence resonance energy transfer (FRET) measurements

CD8 cells were transiently cotransfected with the plasmids encoding the regulatory and the catalytic subunit of PKA fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively (2 μg per each plasmid) using Lipofectin (Invitrogen, San Giuliano Milanese, Italy). Alternatively, cells were cotransfected with actin-YFP (BD Biosciences, San Diego, CA) and ICln-CFP (2 μg of plasmid DNA). To evaluate the specificity of the FRET signals during hypotonicity, cells were cotransfected with actin-YFP (BD Biosciences) and RII-CFP. FRET was measured with an epifluorescence microscope (TE 2000S; Nikon Instruments, Florence, Italy) equipped with a charge-coupled device camera (MicroMax 512BFT; Princeton Instruments, Princeton, NJ) using a DeltaRAM high-speed multiwavelength illuminator for excitation (Photon Technology International, Lawrenceville, NJ) and a beam splitter (Optical Insight, Hercules, MN) on the emission side fitted with a 505DCRX dichroic and two emission filters, D480/30 and D535/40. Excitation was at 430 nm and the dichroic mirror was a 455DRLP. Data were recorded and processed with the MetaMorph/MetaFluor software (Universal Imaging Corp., Downingtown, PA). FRET from CFP to YFP was determined by excitation of CFP (430 nm) and measurement of fluorescence emitted from YFP (535/26 nm).

Immunofluorescence and confocal microscopy analysis

CD8 cells were grown on glass coverslips and fixed with 4% paraformaldehyde in PBS for 20 min and probed with affinity-purified anti-AQP2 antibodies. The protein was visualized with fluorescein-conjugated goat antirabbit IgG (Alexa 488). AQP2 fluorescent signal was detected with a confocal microscope (TCS, SP2; Leica Microsystems, Heidelberg, Germany). The fluorescent distribution and intensity was collected in each confocal plan from the top to the bottom of the selected cell. The data were reorganized in a three-dimensional matrix to have access to any two-dimensional section to see the cell profile in a plane orthogonal to the collected confocal stack.

From the three-dimensional view, a constant region of the same area containing the central part of the nucleus was selected for each cell, and the average value of the intensity of this region for each confocal stack was measured and reported to obtain the fluorescent distribution curve (FDC) plotted as a function of layer depth. Each fluorescent distribution curve was then fitted by two peaks modeled by Gauss line shapes superimposed on a linear background. The data analysis program has been written as an Igor-Pro macro (www.wavemetrics.com), allowing analysis on multidimensional wave, image processing and curve fitting.

In some experiments, CD8 cells were transiently transfected with a plasmid encoding Cdc42-N17 fused with *c-myc* (2 μg of plasmid DNA) using Lipofectin (Invitrogen) and then used to visualize actin cytoskeleton with phalloidin-fluorescein isothiocyanate (100 $\mu\text{g}/\text{ml}$, 45 min). The coverslips were mounted in 50% glycerol in 0.2M Tris-HCl (pH 8.0), containing 2.5% *n*-propyl gallate to retard quenching of the fluorescence. Fluorescent signal was detected with an epifluorescence microscope (TE

2000S; Nikon Instruments) equipped with a charge-coupled device camera (Princeton Instruments MicroMax 512BFT) using a Delta RAM high-speed multiwavelength illuminator for excitation (Photon Technology International, South Brunswick, NJ).

Affinity precipitation of cellular GTP-Cdc42

To evaluate Cdc42 activity, CD8 cells were left either untreated or subjected to hypotonic stress for 10 min at 37 C. Cells were washed with ice-cold buffer containing 150 mM NaCl, 10 mM Tris-buffered saline (pH 7.4) and then lysed in ice-cold radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mM NaCl, 10 mM MgCl_2 , 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ pepstatin A. The cell lysate was clarified by centrifugation at $13,000 \times g$ for 5 min at 4 C and incubated with PAK-GST protein beads (Cytoskeleton Inc., Denver, CO) for 30 min at 4 C. The beads were washed three times with a buffer containing 50 mM Tris-buffered (pH 7.2), 1% Triton, 150 mM NaCl, 10 mM MgCl_2 , 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ pepstatin A. GTP-Cdc42 was eluted by boiling the precipitate in Laemmli buffer for 10 min in the presence of 40 mM dithiothreitol. Bound Cdc42 proteins were detected by Western blotting using a polyclonal antibody against Cdc42.

F-actin cosedimentation assay

F-actin cosedimentation was performed as previously described (22). Briefly, total membrane and cytosol fractions were prepared from CD8 cells. The membrane fraction was resuspended in homogenization buffer at a protein concentration of 2 mg/ml. Protein concentrations of cytosol fractions were equilibrated for protein content and formation of F-actin was initiated by a 50-fold polymerization buffer containing 200 mM MgCl_2 , 4 M KCl, and 100 mM ATP. The samples were incubated for 1 h at 37 C, and F-actin was pelleted by ultracentrifugation for 1 h at 4 C at $150,000 \times g$. The F-actin-containing pellets were rinsed with homogenization buffer. Membrane, cytosol, and F-actin fractions were separated by 13% SDS-PAGE and immunoblotted with LIMK1-specific antibodies.

Transfection of plasmids in cultured cells for patch-clamp experiments

CD8 cells were grown to 70–80% confluence in petri dishes (6 cm diameter) containing DMEM/nutrient mixture F-12 Ham (DMEM/F12) supplemented with 5% (vol/vol) newborn calf serum and then suspended in trypsin 0.025% and seeded in 3.5-cm diameter petri dishes. Cells were grown to 30–40% confluence and transfected with a mixture containing 21 μl of Metafectene TM reagent (Biontex, Martinsried/Planegg, Germany) with pIRES-EGFP or pIRES-EGFP-cdc42-/- (3 $\mu\text{g}/\text{well}$). After 6 h in DMEM/F12, the medium without serum was replaced with medium containing 5% (vol/vol) newborn calf serum. Within 2 d of transfection, cells were used for patch-clamp experiments. To assess the transfection efficiency, EGFP fluorescence was detected by means of a preassembled fluorescent light on an inverted microscope (Zeiss, Jena, Germany).

Patch-clamp technique

The patch-clamp technique and the corresponding statistics were applied as reported (26, 27). In the whole-cell configuration, the resistances of the microelectrodes and of the seals were 2–8 M Ω and 1–2 G Ω , respectively. Signals were filtered at 5 kHz with an eight-pole Bessel filter. In whole-cell configuration, potential differences were expressed as overall potential differences, considering the junction potential and the holding potentials. Voltage steps from –80 to 80 mV were applied (500 msec long increments of 20 mV) under control as well as all bath conditions [hypertonic, then hypotonic, finally hypotonic with 100 μM 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB)]. The bath was grounded with an Ag/AgCl electrode immersed in a 0.5 M KCl agar bridge.

Statistical analysis

All values are expressed as mean $\pm$ SEM. Student's *t* test was used for the statistical analysis. A statistically significant difference was assumed at $P < 0.05$.

Results

Hypotonic shock decreases cell surface expression of AQP2, increases the amount of AQP2 localized intracellularly, and decreases its phosphorylation at ser-256

To investigate the effect of hypotonicity on AQP2 localization, renal CD8 cells were exposed to hypotonic solution for 10 min, and the AQP2 localization as well as any changes in its phosphorylation state was examined.

Immunofluorescence experiments and subsequent analysis by confocal microscopy revealed that after exposure to hypotonic medium the amount of AQP2 localized intracellularly was higher (Fig. 1A). To quantitate this effect, the distribution and intensity of the fluorescence signal was examined in consecutive confocal stacks throughout the cell depth from the top to the basal membrane in a given central region.

Figure 1A (*right panel*) reports a representative distribution of pixel intensity in consecutive confocal stacks. Typically, fluorescence signals distributed in two major peaks defined peak 1, corresponding to the AQP2 signals located in the region between the apical membrane and the nucleus, and peak 2, which appears less prominent and corresponds to AQP2 signals below the nucleus.

The table in Fig. 1 reports the quantization of selected parameters in control cells and cells exposed to hypotonic medium. The ratio between peak 1 and peak 2 areas was significantly increased after hypotonic shock. This was mainly due to an increase in peak 1 area and a slighter decrease in peak 2 area. Quite interestingly, confocal analysis also revealed that the distance of the maximal fluorescence intensity from the nucleus was significantly higher under hypotonic condition (2.95 ± 0.05 vs. 2.162 ± 0.10 μ m of the control). Taken together, these results suggest that the total increase in the amount of AQP2 located intracellularly after hypotonic is due to a relocation of cell surface expressed AQP2 into the subapical cytoplasm.

Western blotting of purified plasma membrane probed with AQP2 antibodies confirmed that hypotonicity decreased cell surface expression of AQP2 (Fig. 1B). Statistical analysis revealed that, compared with control ($100 \pm 12.7\%$), the densitometric signal relative to the AQP2 29-kDa band was significantly reduced by ($55.33 \pm 8.19\%$) after hypotonic shock.

To evaluate whether hypotonic shock causes a decrease of AQP2 abundance because of a protein degradation as recently described for AQP5 (28), cell lysates from control and hypotonic-treated cells were blotted and probed with AQP2 antibodies. Obtained results demonstrated that treatment with the hypotonic solution for 10 min did not alter the total amount of AQP2 protein, most likely indicating that no significant AQP2 protein degradation occurs during hypotonicity within this time period tested (Fig. 1C).

Because a key signal for AQP2 targeting to the plasma membrane in response to vasopressin is the phosphorylation

at ser-256 (phospho-AQP2) by PKA, we next evaluated the amount of phospho-AQP2 after hypotonic shock.

Western blotting analysis using a phosphospecific antibody specifically recognizing AQP2 phosphorylated at ser-256, revealed that hypotonic stress was paralleled by a strong decrease in phosphorylated AQP2 ($63.6 \pm 9.3\%$ vs. control $100 \pm 7.9\%$, $n = 5$) (Fig. 2A). This effect was also confirmed in native principal cells. Isolated rat kidney collecting ducts were subjected to the same experimental protocol, homogenized, and probed with phosphospecific AQP2 antibodies (Fig. 2B). The specificity of phospho-AQP2 antibodies was tested using a cell lysate from the RC.SV3 rabbit collecting duct cell line stably transfected with S256A AQP2 (24). Antiphospho-AQP2 antibodies failed to recognize the S256A AQP2, whereas the anti-AQP2 detected the unphosphorylated AQP2 form expressed in this cell line (Fig. 2C, *left panel*). The specificity of antiphospho-AQP2 antibodies was further confirmed by ELISA. Phospho-AQP2 antibodies recognized phosphopeptide in a dose-dependent manner. Phospho-AQP2 antibodies did not reacted with AQP2 peptide confirming the antibody specificity (Fig. 2C, *right panel*).

Because cAMP-dependent PKA is committed to phosphorylate AQP2, we next examined the spatiotemporal intracellular dynamics of cAMP using the FRET technique in living cells. FRET is a process in which energy from a fluorescent donor molecule is transferred to a fluorescent acceptor. The fluorescent emission of the acceptor is enhanced by the excitation of the donor molecule. The regulatory and catalytic subunits of PKA, fused with the respective green fluorescent protein mutants, were used for measuring changes in FRET that correlate with changes in intracellular cAMP levels (Fig. 3A). Using this probe, we can estimate changes in the intracellular cAMP concentration. A decrease in intracellular cAMP concentration is assumed if both PKA regulatory and PKA catalytic subunits are associated, an event measured by the FRET technique.

For this reason, the regulatory and catalytic subunits of PKA were transiently cotransfected in CD8 cells with plasmids encoding RII-CFP and catalytic PKA-subunit-YFP. Figure 3B shows a representative cell cotransfected with plasmids encoding RII-CFP and C-YFP in basal (0 sec) and after hypotonic stress (1000 sec). The FRET signal (ratio 480:535) is depicted in pseudocolor. The time course of the mean values of FRET signal ratios calculated in 18 single cells are reported in Fig. 3C. Hypotonic treatment decreased the FRET signal by 0.893 ± 0.009 within 475 sec, which means that PKA activity decreases during hypotonic shock. This result might explain the observed decrease in the total amount of phospho-AQP2.

Actin remodeling during hypotonic shock

An important consequence of cell swelling is the reorganization of the actin cytoskeleton. In CD8 cells, the actin cytoskeleton underwent a deep remodeling during cell swelling, consisting in the loss of stress fibers and the formation of F-actin patches and numerous microspikes at the cell border (Fig. 4). The actin cytoskeleton is a dynamic structure under the control of the monomeric G protein of the Rho family (Rho, Rac and Cdc42), controlling a variety of pro-

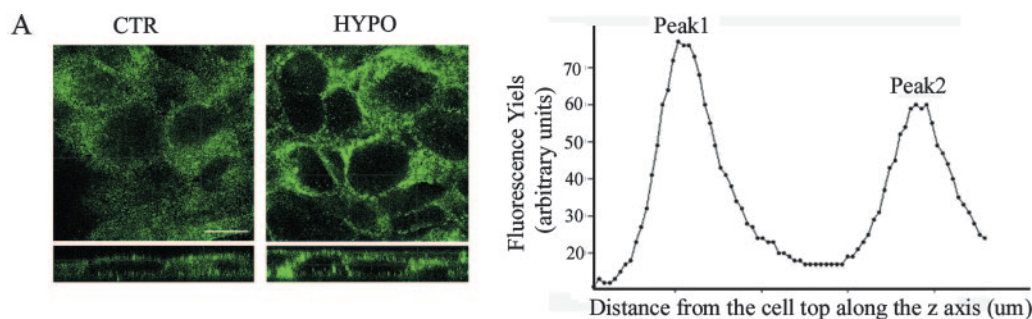


Table 1 Analysis of AQP2 related fluorescence distribution by confocal microscopy

Fluorescence parameters	CTR n=26	HYPO n=27
Peak1/Peak2 Ratio of respective areas arbitrary units	1,963±0,18	4,170±0,62*
Peak1/total cell area arbitrary units	0,315±0,11	0,479±0,02*
Peak2/total cell area arbitrary units	0,127±0,005	0,159±0,014*
Maximal fluorescent intensity from the nucleus towards the apical membrane (μm)	2,162±0,10	2,95±0,05*

Data are expressed as means ± SE *P<0,05 (Student t-test)

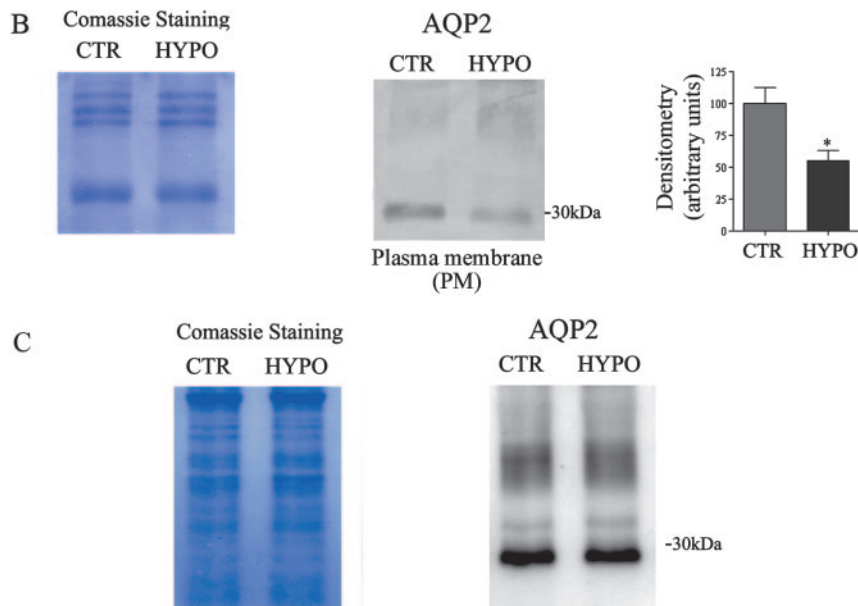


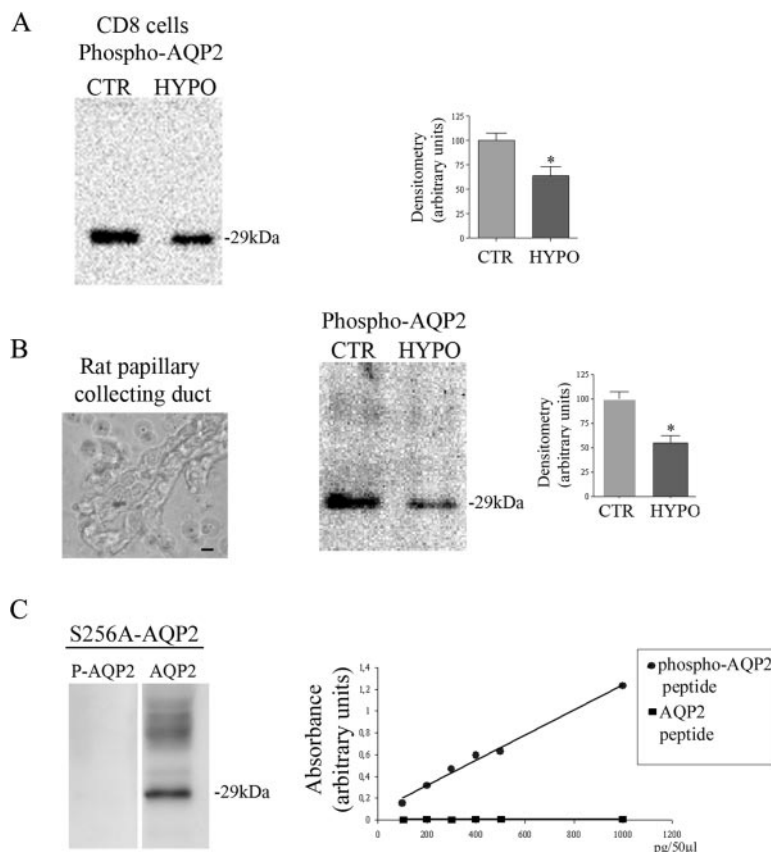
FIG. 1. Localization of AQP2 in CD8 cells. A, *Left*, CD8 cells were left untreated or incubated with hypotonic solution for 10 min. After fixation, cells were immunostained with anti-AQP2 antibody (1:500). Fluorescence was visualized with a confocal microscope. A representative xy plan and xz reconstruction is visualized for each condition. Bar, 10 μ m. A, *Right*, Representative distribution of pixel intensity in consecutive confocal stacks from a cell stained with anti-AQP2 antibody. Fluorescence signals distributed in two major peaks defined peak 1, corresponding to the AQP2 signals located in the region between the apical membrane and the nucleus, and peak 2, which is less prominent and corresponds to AQP2 signals below the nucleus. B, AQP2 abundance in plasma membrane fractions. Plasma membranes (PM) were obtained using Qproteome plasma membrane kit according to the manufacturer's instructions. Equal amounts of proteins from control (CTR) and hypotonic (HYPO)-treated cells were immunoblotted and probed with anti-AQP2 antibody. On the *right*, the statistical analysis of signal intensity relative to the immunodetected bands is reported. Values are expressed as means $\pm$ SE (n = 4; *, $P < 0.05$). C, Total AQP2 content after hypotonic shock. Equal amounts of proteins of lysates from control and hypotonic-treated cells were immunoblotted and probed with anti-AQP2 antibody. A representative Western blotting performed a total of six times is shown. Hypotonic treatment did not modify the total amount of immunodetected AQP2.

cesses including AQP2 trafficking (8, 24, 29–31). In previous studies performed in rat-1 fibroblast, it has been shown that inhibition of all members of the Rho family by toxin B abolished hypotonic-dependent actin reorganization, whereas C3 toxin, which selectively inhibits RhoA protein, did not prevent actin remodeling during hypotonicity, indicating that RhoA activity is not involved in this process. In addition,

hypotonic stimulation rearranges the actin cytoskeleton and induces the formation of membrane protrusion via the activation of Cdc42 (32).

To investigate the signals regulating actin cytoskeleton remodeling in renal cells, CD8 cells were transiently transfected with a plasmid encoding for the constitutively inactive Cdc42 (Cdc42-N17) fused with *c-myc*. Cdc42-N17 expressing

FIG. 2. AQP2 phosphorylation during hypotonicity. A, CD8 cells were left under basal conditions (CTR) or subjected to hypotonic stress (HYPO) for 10 min. After homogenization, equal amounts of proteins were separated by SDS-PAGE and immunoblotted using a phospho-AQP2 antibody. The mean values of densitometric profiles of the bands is shown on the *right* (means $\pm$ SE, $n = 5$; *, $P < 0.05$). Hypotonicity caused a significant decrease in phospho-AQP2. B, *Left*, Phase-contrast micrograph of papillary collecting duct tubule (bar, 10 μ m). B, *Right*, Rat papillary collecting duct tubules were left untreated or subjected to hypotonic solution. Collecting duct tubules were then lysated and used to evaluate the amount of phospho-AQP2. Values are means $\pm$ SE ($n = 6$; *, $P < 0.05$). C, *Left*, The RC.SV3 rabbit collecting cells stably transfected with S256A-AQP2 were lysed and immunoblotted with either antiphospho-AQP2 antibodies or anti-AQP2 antibodies. C, *Right*, The specificity of antiphospho-AQP2 antibodies was confirmed by ELISA. Phospho-AQP2 antibodies recognized phosphopeptide in a dose-dependent manner (●). Phospho-AQP2 antibodies did not react with AQP2 peptide, confirming the antibody specificity (■).



cells were visualized by detecting *c-myc* tags by immunofluorescence 24 h after transfection.

In hypotonic-treated Cdc42-N17 expressing CD8 cells, the typical membrane protrusion observed in transfected cells were no longer visible (Fig. 4). This suggests that Cdc42 might be involved in the regulation of the actin cytoskeleton during cell swelling. To verify whether Cdc42 activation occurs during cell swelling, the total amount of active Cdc42 (GTP-Cdc42) in basal and after hypotonic exposure for 10 min was measured by pull-down assay. Compared with the control, hypotonicity resulted in a significant increase in the amount of active GTP-Cdc42 ($186.8 \pm 10.35\%$ vs. control $100 \pm 12.7\%$ $n = 4$), confirming that this monomeric of Rho family G protein is activated and is involved in the formation of microspikes at the cell border (Fig. 5).

A known downstream effector of Cdc42 is LIM kinase (LIMK, in which LIM is an acronym of the three gene products Lin-11, Isl-1, and Mec-3), existing as two isoforms (LIMK1 and LIMK2), which exert their kinase activity by inactivating the ADP/depolymerizing factor, cofilin. Cofilin phosphorylation results in a strong stabilization of actin filaments (33). Interestingly, using an F-actin cosedimentation assay, we found that hypotonicity caused a redistribution of LIMK1 in a particulate fraction enriched in a cellular membrane (P) paralleled by a decrease in the soluble fraction (S) (P/S $315.5 \pm 39.4\%$ vs. control $100 \pm 11.61\%$; $n = 3$) (Fig. 6A). Quantification of this effect demonstrated that upon hypotonic treatment, LIMK1 abundance increases in the F-actin enriched fraction by $246.6 \pm 8.5\%$, compared with the control

condition ($100 \pm 7.15\%$; $n = 3$) (Fig. 6B). No such effect was observed for LIMK2 (not shown).

Consistent with cofilin being the protein target of LIMK1, cell fractionation experiments revealed an increase in phosphorylated cofilin associated with the membrane under hypotonic treatment ($185.4 \pm 23.82\%$ vs. control $100 \pm 4.88\%$; $n = 4$) (Fig. 7).

Hypotonic shock causes transposition of ICln to the cell membrane and activation of Cl^- conductance

ICln is a multifunctional protein, preferentially located in the cytosol, that is essential for the generation of ion currents activated during regulatory volume decreases after cell swelling (16). Furthermore, it has been shown that the subcellular localization of ICln in distal tubule cells is dependent on the diuretic state and that the ICln protein is associated with the actin system.

To investigate whether hypotonicity stimulates ICln membrane association in renal CD8 cells, cell fractionation experiments were performed from control and hypotonic-treated cells. Western blotting of the fractions revealed that in control cells the bulk of the immunoreactive ICln was localized in the soluble fraction. Incubation in hypotonic solution for 10 min resulted in a significant increase in ICln abundance in the fraction enriched in cellular membrane, suggesting a functional role of this protein during swelling (Fig. 8, A and B). The essential role of ICln in RVD is further underlined by the fact that knocking down the ICln protein

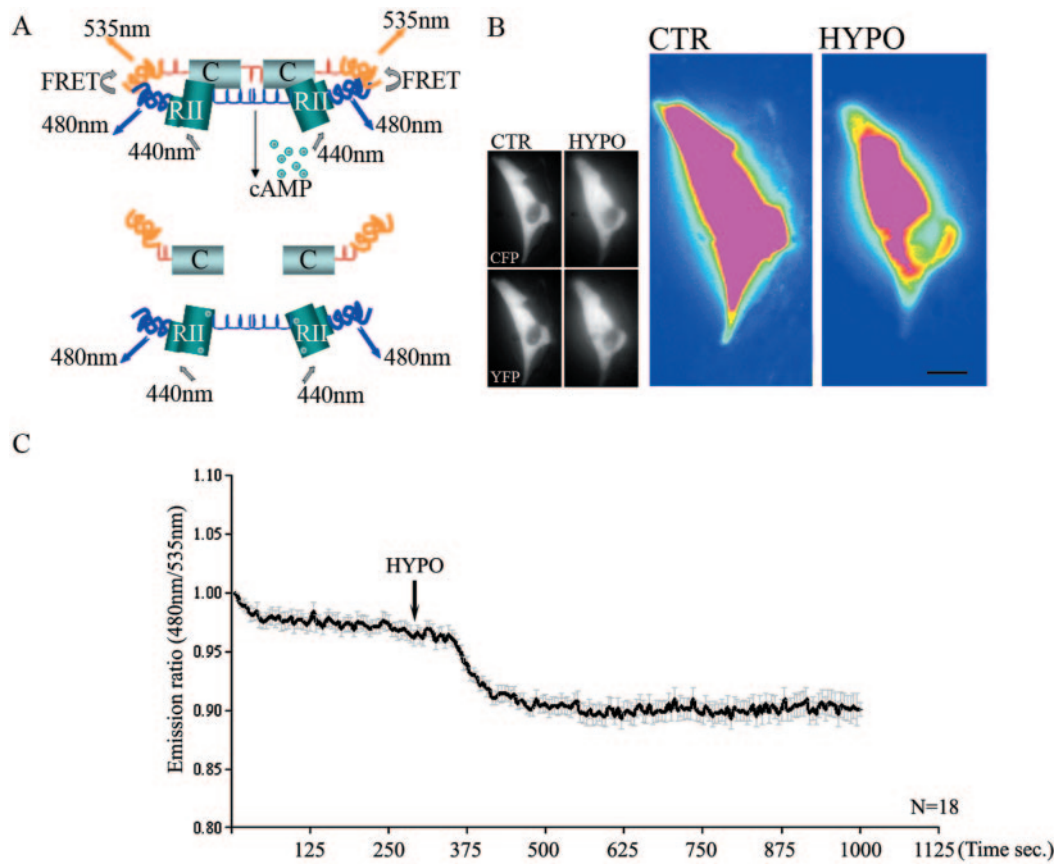


FIG. 3. Evaluation of PKA activity by FRET analysis. A, Schematic model showing regulatory (R) and catalytic (C) subunits of PKA fused with CFP and YFP used to monitor PKA activity. Low concentrations of cAMP are associated with a decrease in PKA activity, which results in a specific interaction between catalytic and regulatory subunits. When the cAMP level increases, regulatory subunits detach from the active catalytic subunits, impairing the fluorescence energy transfer process from the donor (CFP) to the acceptor (YFP). B, Representative cell cotransfected with plasmids encoding RII-CFP and C-YFP in basal (CTR; 0 sec) and after hypotonic stress (HYPO; 1000 sec) (*bar*, 10 μ m). The FRET signal (ratio 480:535) is depicted in pseudocolor. C, Quantitative evaluation of hypotonicity on FRET signal ($n = 18$ cells).

in 3T3 fibroblasts by the use of antisense oligodeoxynucleotides leads to a marked reduction in the swelling-induced ion current (20, 34). Furthermore, the overexpression of ICln in the same cells is paralleled by an increase in the swelling-induced ion currents.

The activation of swelling-induced ion currents was also confirmed in this study by patch clamping, in a whole-cell configuration, CD8 cells (Fig. 9A). For this reason, CD8 cells were exposed to hypotonic solution for 25 min to attain maximum activation of swelling-induced ion currents (Fig. 9, C and E). The elicited chloride currents showed an outward rectification and inactivation at positive voltages over +60 mV and were blocked by 10–4 M NPPB (Fig. 9, C–E), which is in agreement with ICl, swell characteristics determined in other cellular systems (18). The Cl[−] currents measured in hypotonic solution in CD8 cells were significantly different from those observed in hypertonic conditions. CD8 cells expressing pIRES-EGFP also exhibit significant differences in current ($P < 0.05$) between the hypotonic and hypertonic conditions (10.34 ± 2.86 pA/pF, $n = 7$ at +75.4 mV and -6.80 ± 1.85 pA/pF, $n = 7$ at −84.6 mV, as against 1.74 ± 0.68 pA/pF, $n = 7$ at +75.4 mV and -2.26 ± 0.92 pA/pF, $n = 7$ at −84.6 mV, $P < 0.05$).

Interestingly, expression of the constitutively inactive

Cdc42 (Cdc42-N17) abolished hypotonicity-induced anion currents (Fig. 9, F–J), indicating that actin remodeling under the regulatory control of Cdc42 is essential for the activation of this anion conductance. Indeed, ICln activation has been suggested to occur through modulation by cytoskeleton and caveole, which might mediate its transposition to the cell plasma membrane (5).

The dynamics of ICln and actin interaction upon hypotonic shock were next explored by several experimental strategies. Using an extraction procedure with Triton X-100 buffer, which preserves the cytoskeleton and the cytoskeleton-associated proteins, we found that, relative to the control, the amount of actin-dissociated ICln was significantly decreased upon hypotonic treatment, suggesting that a significant amount of ICln redistributes to an F-actin-enriched fraction (Fig. 10A).

Coimmunoprecipitation experiments confirmed that the amount of actin coimmunoprecipitated with ICln increased by $145.7 \pm 4.75\%$, compared with control cells ($100 \pm 3.7\%$, $n = 3$), providing direct biochemical evidence for an increased interaction between ICln and actin during hypotonicity (Fig. 10B).

Real-time dynamic interaction between actin and ICln was eventually evaluated in living cells by the FRET technique.

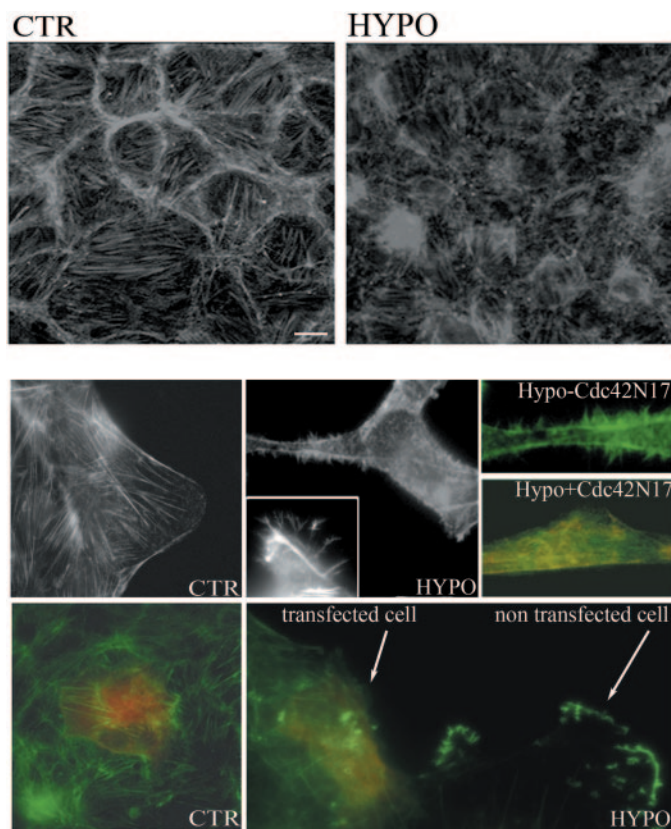


FIG. 4. Effect of hypotonicity on actin remodeling. CD8 cells were left untreated (CTR) or incubated with hypotonic solution for 10 min (HYPO). Alternatively, cells were transiently transfected with the constitutively dominant-negative Cdc42 (Cdc42N17) tagged with *c-myc* and subjected to hypotonic treatment. F-actin was stained with fluorescein isothiocyanate-conjugated phalloidin and visualized by an epifluorescence microscopy, whereas the *c-myc*-positive cells were first incubated for 1 h with monoclonal anti *c-myc* antibody and then with rabbit antimouse TRITC-conjugated antibody. The pictures were subjected to 40 cycles of three-dimensional deconvolution using Autodeblur software (Universal Imaging Corp.). Hypotonicity induces a strong actin reorganization and promotes the formation of cell membrane protrusion. The expression of Cdc42N17 abolished the formation of actin enriched structures at the cell cortex.

CD8 cells were cotransfected with plasmids, encoding, respectively, the fusion protein ICln-CFP and actin-YFP, and FRET measurements were performed 24 h after transfection. FRET (ratio 480:535 nm) signals were recorded every 5 sec. Exposure to hypotonic solution caused an increase in the interaction between ICln and actin resulting in a decrease of FRET ratio (480:535 nm) by 0.876 ± 0.02 ($n = 18$) (*vs.* control 1), reaching the lower value after 500 sec (Fig. 11A). This effect was reversible upon perfusion with an isotonic solution. To confirm the specificity of the ICln and actin interaction, FRET experiments were performed cotransfecting CD8 cells with actin-YFP and RII-CFP, which are not supposed to interact. Exposure to hypotonic solution did not change the FRET ratio (480:535 nm) (Fig. 11B).

Discussion

Collecting duct epithelial cells have to counteract the cell swelling occurring during the transition from antidiuresis to

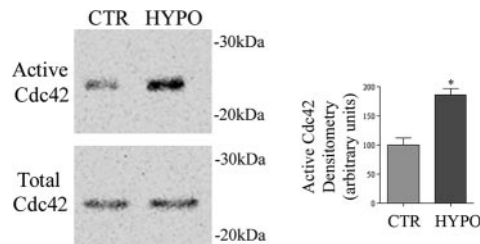


FIG. 5. Effect of hypotonicity on Cdc42 activity. Affinity precipitation of cellular GTP-Cdc42 by pull-down assay was performed in CD8 cells left untreated (CTR) or incubated with hypotonic solution for 10 min (HYPO). The lysates from each condition were incubated with 20 μ g PAK-GST protein beads (Cytoskeleton Inc.). GTP-Cdc42 was precipitated and subjected to Western blot studies with anti Cdc42 antibodies. Results are representative of three independent experiments with similar results. Densitometric analysis (means $\pm$ SE, $n = 3$) of the GTP-Cdc42 bands relative to the control is shown on the right (*, $P < 0.05$).

diuresis, with a variety of mechanisms for responding to volume change. Because changes in inner medulla osmolarity occur within minutes, it is of critical physiological relevance to identify the solute and water transporters modified during this event. Despite its importance, very few studies have focused on the synchronized modification of both transport entities, *i.e.* solutes and water after cell swelling.

In the present study, we describe several alterations occurring in AQP2-expressing collecting duct cells in response to exposure to a hypotonic medium. Those alterations can be summarized as follows: 1) an increase in the total amount of

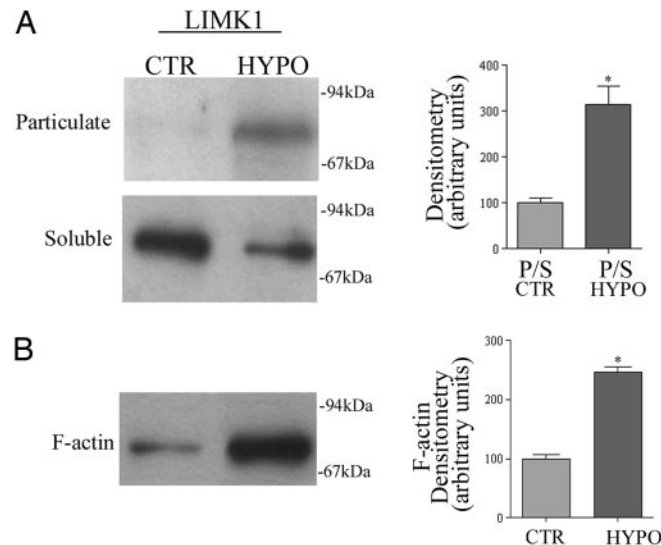


FIG. 6. Effect of hypotonicity on LIMK1 subcellular localization. A, Equal amount of proteins (30 μ g/lane) from particulate (P; 150,000 $\times$ g pellet) and soluble (S; 150,000 $\times$ g supernatant) fractions from control (CTR) and hypotonic-treated (HYPO) cells were separated by gel electrophoresis and immunoblotted with anti-LIMK1 antibodies. In hypotonic-treated cells, the ratio of LIMK1 abundance in the P fraction and in the S fraction (P/S) was significantly higher, compared with the control. Densitometric analysis (means $\pm$ SE, $n = 3$; *, $P < 0.05$) of LIMK1 bands is shown on the right. B, Cytosolic fractions from CD8 cells were prepared as described in *Materials and Methods*. Equal amounts of F-actin-enriched fractions (30 μ g/lane) were immunoblotted with specific antibody against LIMK1. Densitometric analysis (mean $\pm$ SE, $n = 3$) of LIMK1 bands are shown on the right (*, $P < 0.01$).

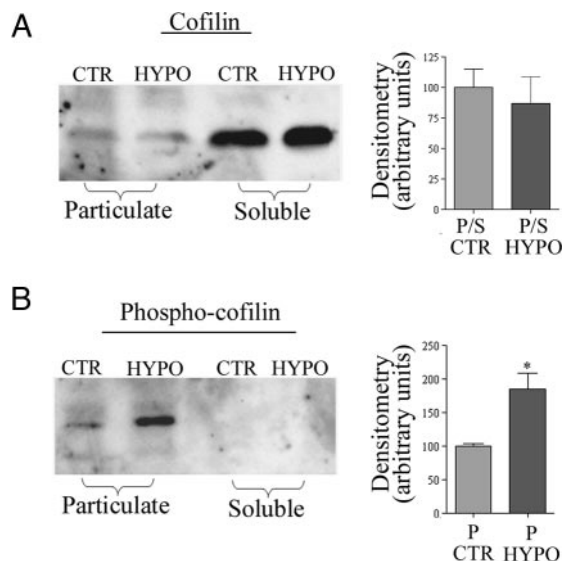


FIG. 7. Subcellular distribution of cofilin upon hypotonic stress. A, Equal amount of proteins ($30 \mu\text{g}/\text{lane}$) from particulate (P; $150,000 \times g$ pellet) and soluble (S; $150,000 \times g$ supernatant) fractions from control (CTR) and hypotonic-treated (HYPO) cells were separated by gel electrophoresis and immunoblotted with anti cofilin antibodies. In hypotonic-treated cells, the ratio of cofilin abundance in the P and S fractions (P/S) was similar to that observed in the control condition. Densitometric analysis (mean $\pm$ SE, $n = 4$) of cofilin bands is shown on the right. B, The particulate and the soluble fractions from control and hypotonic-treated cells were probed with anti P-Ser 3-cofilin antibody. A specific band corresponding to the expected molecular weight of P-Ser 3-cofilin was detectable only in the particulate fraction and was significantly higher upon hypotonic shock. Densitometric analysis (mean $\pm$ SE, $n = 4$) of P-Ser 3-cofilin bands is shown on the right (*, $P < 0.05$).

AQP2 localized intracellularly, associated with a decrease in phosphorylation at ser-256; 2) profound actin remodeling controlled by Cdc42 signaling; and 3) transposition of ICln from the cytosol to the plasma membrane, associated with activation of a Cl^- current. It is highly predictable that all these effects may contribute to regulatory volume decrease after hypotonicity-induced cell swelling in renal medulla.

The question of whether AQPs are involved in cell volume regulation has not been widely investigated, although it is

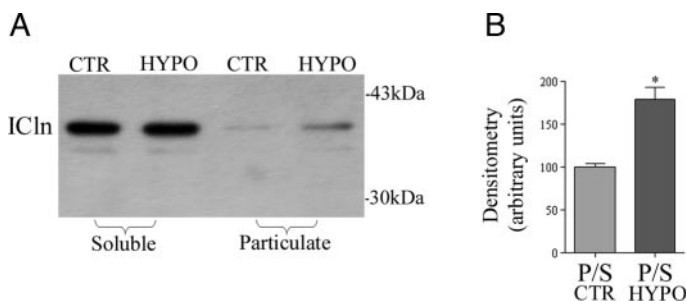


FIG. 8. Cellular distribution of ICln in CD8 cells. A, Cell fractionation experiments were performed from control (CTR) and hypotonic-treated (HYPO) cells. Equal amounts of proteins were immunoblotted with ICln antibody specifically recognizing the ICln protein at 36 kDa. In hypotonic-treated cells, the ratio of ICln abundance in the P fraction to that in the S fraction (P/S) was higher than in the control condition. B, Densitometric analysis (mean $\pm$ SE, $n = 4$) of ICln bands is shown on the right (*, $P < 0.05$).

reasonable to speculate such a role because volume regulation involves solute movements that drive osmotic water transport (35).

Indeed, most of the available data concern the response of renal medullary cells to long-term adaptation to osmotic stress (36, 37). In immortalized mouse collecting duct principal cells [mpkCCD(c14)], increased extracellular tonicity first decreased AQP2 gene expression after 3 h, whereas 24 h exposure of cells enhanced AQP2 expression (38). Moreover, previous studies have shown that chronic (64 h) adaptation to hypertonicity in Madin-Darby canine kidney cells resulted in a basolateral instead of apical insertion of AQP2 in response to forskolin (39).

Besides the long term adaptation, it is, however, of great interest to investigate how renal cells react to rapid change in extracellular environment osmolality.

Osmotic equilibration is in fact a very rapid process in most cells (less than 1 min, often in seconds) and because volume regulation occurs over many minutes, water permeability should not be rate limiting in volume regulation. Few studies have raised the possibility that AQPs play a role in cell volume regulation (40, 41).

In particular, regarding the involvement of the AQP2 water channel in volume regulation, Ford *et al.* (42) reported that in cortical collecting duct cells displaying a constitutive expression of AQP2 in the apical plasma membrane, hypotonic shock induces RVD more rapidly than in cortical collecting duct cells not expressing AQP2. This suggests that the presence of a water channel in the cell membrane may be critical for the rapid activation of RVD mechanisms. In a more recent paper, the same authors demonstrated that the rate of osmosis produced by a hypotonic shock depends on the gradient direction only in the presence of apical AQP2 (42).

We report here that, in response to short exposure to a hypotonic medium, the total amount of AQP2 localized intracellularly is increased in CD8 cells, a well-characterized collecting duct cell line in which AQP2 is inserted into the apical membrane in response to cAMP-elevating agents (43). Quite interestingly, hypotonic shock causes a decrease in the total amount of AQP2 phosphorylated at ser-256, consistent with the observed decrease in PKA activity during cell swelling (Fig. 3, FRET experiments).

Our experimental data suggest that hypotonicity causes an increase in AQP2 internalization through a reduction in constitutive recycling (either decreased insertion or increased retrieval). This is a short-term response occurring within a few minutes of the hypotonic shock.

A straightforward explanation for this effect is that diversion of AQP2 from the apical side on which an osmotic shock takes place during the transition from antidiuresis to diuresis may be a protective mechanism limiting apical water entry, thus reducing cell swelling. In this context, the reduction in the total amount of p-AQP2 renders less efficient the mechanism of AQP2 insertion and probably of AQP2 retention in the apical membrane. Interestingly, a very recent finding demonstrated that the water channel AQP5 expressed in the epithelia of lung, cornea, and various secretory glands, sites in which extracellular osmolality is known to fluctuate, is reduced in response to extracellular hypotonicity, and this

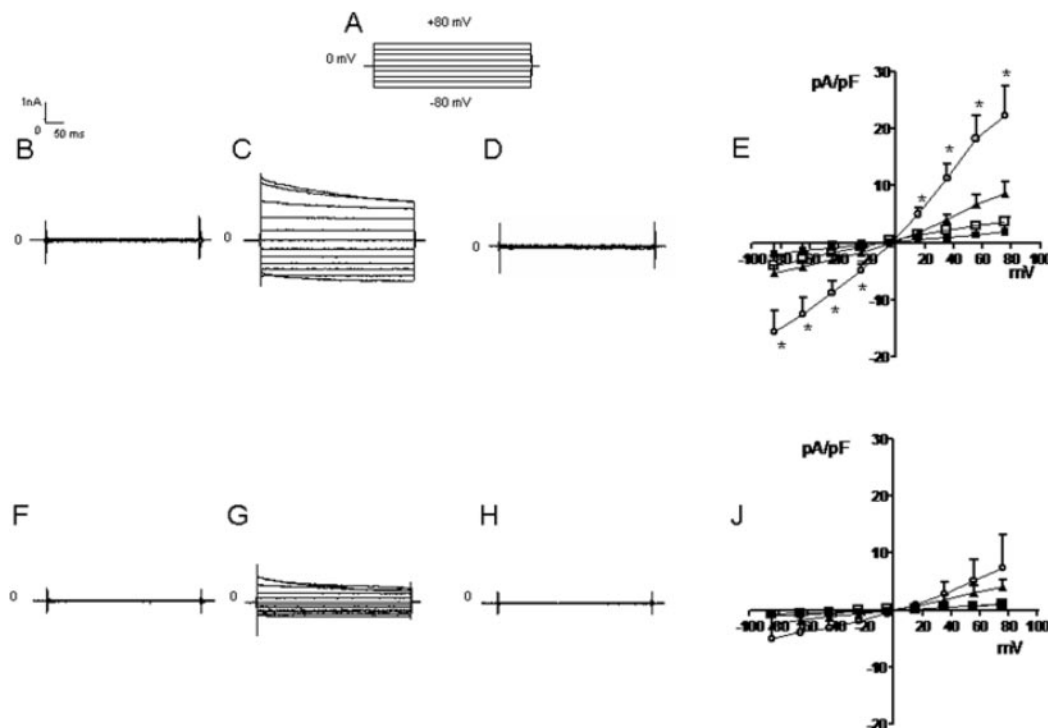


FIG. 9. Effects of osmolality on Cl^- currents in CD8 cells, patch-clamp experiments in whole-cell configuration. A, Stimulation protocol. B, Whole-cell currents in hypertonic medium. C, Whole-cell currents in hypotonic medium after 25 min. D, Whole-cell currents in hypotonic medium with 10^{-4} M NPPB. E, CD8 cell current-voltage (I/V) relationships in hypertonic, hypotonic medium at different times or in the presence of 10^{-4} M NPPB ($n = 21$). F–J, Osmolarity effects on Cl^- currents in CD8 cells transfected with Cdc42N17. F, Whole-cell currents in hypertonic medium. G, Whole-cell currents, 25 min in hypotonic medium. H, Whole-cell currents in hypotonic medium with 10^{-4} M NPPB. J, Current-voltage (I/V) relationships in hypertonic, hypotonic medium at different times or in the presence of 10^{-4} M NPPB ($n = 5$). ■, Hypertonic medium; ▲, 7 min in hypotonic medium; ○, 25 min in hypotonic medium; □, hypotonic medium with 10^{-4} M NPPB. *, $P < 0.05$, compared with hypertonic condition.

effect can be mediated by TRPV4 (28). Moreover, in salivary glands it has been found that AQP5 is required for the activation of TRPV4 by hypotonicity and that both transporters control RVD in salivary glands (44).

If AQPs are involved in volume regulation, then other mechanisms would need to be involved, such as interactions between AQPs and solute transporters involved in the primary volume regulatory response. On this line Sugiya and Matsuki (45) suggest that AQP5 might act as an osmotic sensor in the secretory granules of the parotid gland, and they hypothesize that a balance of water permeation via AQP5 and Cl^- conductance is necessary for secretory granule volume regulation.

We show here a synchronized modification of both AQP2 and the multifunctional protein ICln, a protein essential for the generation of ion currents activated during RVD after cell swelling (17). In CD8 cells, we found that ICln translocates from the cytosol to the cell membrane in CD8 cells after cell swelling.

ICln is water soluble and resides primarily in the cytosol, whereas a small fraction of ICln is associated with the cell membrane (46). It has been shown that during a hypotonic challenge, ICln migrates from the cytosol toward the cell membrane in NIH 3T3 fibroblasts, Madin-Darby canine kidney cells, and LLC-PK1 epithelial cells (15), suggesting a role for ICln in cell volume regulation (47). Interestingly, in the inner medulla and papilla of rats infused with the loop diuretic furosemide, which led to a decrease in urine osmo-

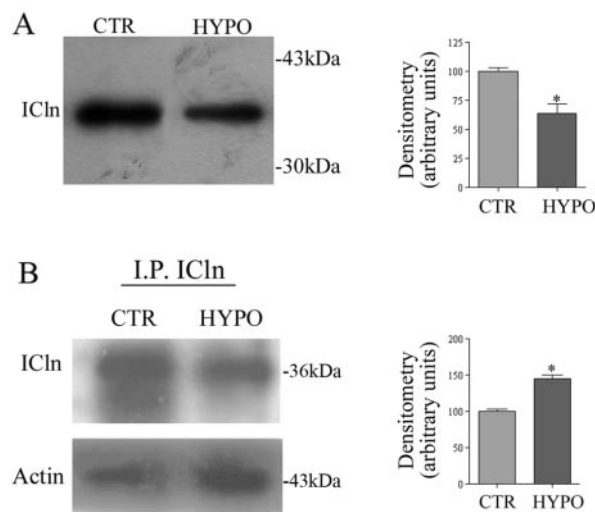


FIG. 10. Dynamics of ICln association with actin cytoskeleton. A, The association of ICln with actin filaments was evaluated by its solubility in Triton X-100, which preserves cytoskeleton and cytoskeleton-associated proteins. Densitometric analysis (means $\pm$ SE, $n = 4$ of immunoreactive bands is shown on the right (*, $P < 0.05$). B, The direct interaction between actin and ICln was analyzed by coimmunoprecipitation (I.P.) experiments. Left panel, Representative of three independent experiments showing that hypotonicity increases the association of ICln and actin. Right panel, Densitometric analysis of immunodetected bands normalized with the total amount of the immunoprecipitated ICln protein (means $\pm$ SE, $n = 3$; *, $P < 0.05$). CTR, Control; HYPO, hypotonic treated.

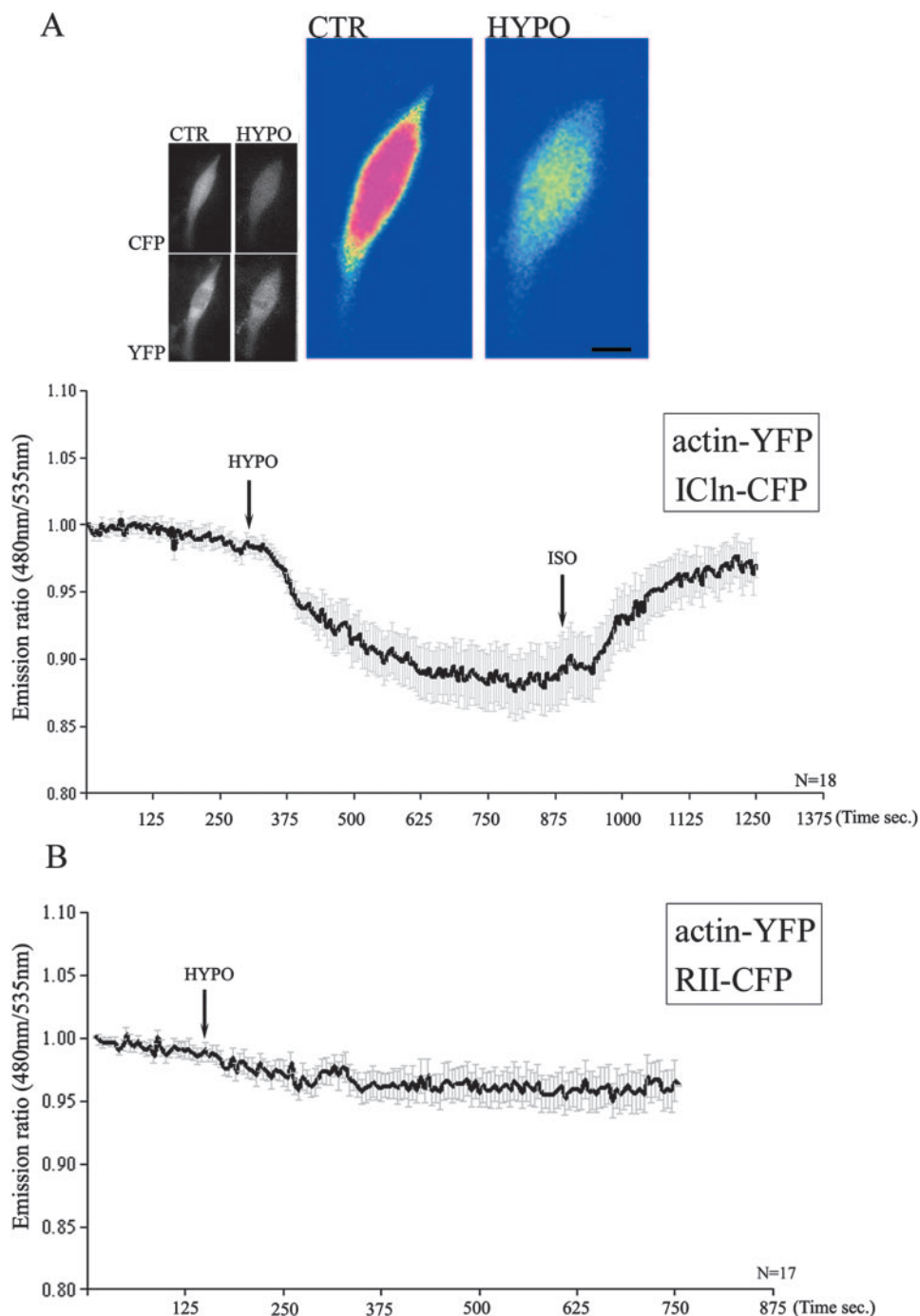


FIG. 11. A, Evaluation of the dynamic interaction between actin and ICln during hypotonicity. A representative cell cotransfected with plasmids encoding ICln-CFP and actin-YFP in basal (CTR; 0 sec) and after hypotonic stress (HYPO; 900 sec) is shown. FRET signal (ratio 480:535) is depicted in pseudocolor. On the bottom is shown the quantitative evaluation of hypotonicity on FRET signal during hypotonic shock ($n = 18$ cells) clearly indicating that hypotonic treatment causes a marked increase in actin-ICln interaction. Indeed this effect was reversible upon perfusion with an isotonic solution. B, Hypotonic effect on FRET signal measured in cells cotransfected with plasmids encoding RII-CFP and actin-YFP ($n = 17$ cells).

larity due to the transition to a diuretic state, ICln is preferentially sorted to the apical and basolateral cell membranes (15). This suggests a primary role for ICln in volume regulation to counteract cell swelling. It is likely that the activation of the chloride current associated with ICln transposition to the cell membrane described in the present study in CD8 cells is the physiological response to hypotonic shock in collecting duct cells.

As mentioned, ICln is mainly a cytosolic protein and regulatory factors are certainly necessary for the membrane association of ICln. It has been suggested that interactions of

ICln with cytoskeletal proteins such as actin (46, 48, 49) and erythrocyte protein 4.1 (50) may be important for ICln activation. Indeed, a direct ICln-actin interaction has been shown to occur in CD8 cells during hypotonicity-induced cell swelling. Reorganization of the F-actin cytoskeleton has been shown to be an important consequence of cell swelling in many cell types (32). Interestingly, we report here a detailed analysis of actin remodeling regulated by the small GTP-binding protein Cdc42, consisting in the appearance of membrane protrusions at the cell borders in which actin patches are concentrated. These actin-rich membrane spikes may be

active zones in which ICln is recruited through interaction with actin, and anion conductance is activated. Consistent with this hypothesis, the expression of dominant-negative Cdc42 prevents the formation of such structures after hypotonic shock and abolishes the chloride currents. Moreover, in this study, we have also identified the downstream effector of Cdc42, the kinase LIMK1 that in turn phosphorylates cofilin, a potent regulator of actin filament dynamics, which binds and depolymerizes actin when nonphosphorylated (51–53).

In summary, the results of the present study indicate that in medullary collecting duct cells, exposure to hypotonic fluid causes a rapid swelling, deep F-actin remodeling and activation of a chloride current associated with translocation of ICln to the plasma membrane, likely through a dynamic interaction with F-actin. Interestingly, under this condition, the total amount of AQP2 localized intracellularly increases with a concomitant decrease in p-AQP2.

The diversion of AQP2 from the apical side, on which an osmotic gradient is generated during the transition from antidiuresis to diuresis, may be a protective mechanism limiting apical water entry, and thus reducing cell swelling. On the other hand, Cl[−] ion efflux (and probably other osmolites) followed by water efflux likely occurring mainly from the basolateral side (probably through AQP3 and AQP4) may contribute to RVD after hypotonicity-induced cell swelling in renal medulla.

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