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Extracellular calcium antagonizes forskolin-induced aquaporin 2 trafficking in collecting duct cells

Giuseppe Procino,[1] Monica Carmosino,[1] Grazia Tamma, Sabine Gouraud, Antonia Laera, Daniela Riccardi, Maria Svelto, and Giovanna Valenti

Department of General and Environmental Physiology, University of Bari, Bari, Italy; Centro di Eccellenza di Genomica Comparata, University of Bari, Italy; and School of Biological Sciences, University of Manchester, Manchester, United Kingdom

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Background. Urinary concentrating defects and polyuria are the most important renal manifestations of hypercalcemia and the resulting hypercalciuria.

In this study, we tested the hypothesis that hypercalciuria-associated polyuria in kidney collecting duct occurs through an impairment of the vasopressin-dependent aquaporin 2 (AQP2) water channel targeting to the apical membrane possibly involving calcium-sensing receptor (CaR) signaling.

Methods. AQP2-transfected collecting duct CD8 cells were used as experimental model. Quantitation of cell surface AQP2 immunoreactivity was performed using an antibody recognizing the extracellular AQP2 C loop. Intracellular cyclic adenosine monophosphate (cAMP) accumulation was measured in CD8 cells using a cAMP enzyme immunoassay kit. To study the translocation of protein kinase C (PKC), membranes or cytosol fractions from CD8 cells were subjected to Western blotting using anti-PKC isozymes antibodies. The amount of F-actin was determined by spectrofluorometric techniques. Intracellular calcium measurements were performed by spectrofluorometric analysis with Fura-2/AM.

Results. We demonstrated that extracellular calcium ($Ca^{2+}{}_o$) (5 mmol/L) strongly inhibited forskolin-stimulated increase in AQP2 expression in the apical plasma membrane. At least three intracellular pathways activated by extracellular calcium were found to contribute to this effect. Firstly, the increase in cAMP levels in response to forskolin stimulation was drastically reduced in cells pretreated with $Ca^{2+}{}_o$ compared to untreated cells. Second, $Ca^{2+}{}_o$ activated PKC, known to counteract vasopressin response. Third, quantification of F-actin demonstrated that $Ca^{2+}{}_o$ caused a nearly twofold increase in F-actin content compared with basal conditions. All these effects were mimicked by a nonmembrane permeable agonist of the extracellular CaR, Gd^{3+}.

Conclusion. Together, these data demonstrate that extracellular calcium, possibly acting through the endogenous CaR, antagonizes forskolin-induced AQP2 translocation to the apical plasma membrane in CD8 cells. In hypercalciuria, this mechanism might blunt water reabsorption and prevent further calcium concentration, thus protecting against a potential risk of urinary calcium-containing stone formation.

Vasopressin acutely regulates the water permeability of the kidney collecting duct by inserting aquaporin 2 (AQP2) in intracellular vesicles to the luminal plasma membrane. As a consequence, water is reabsorbed, leading to urine concentration [1–4]. In several pathologic conditions, characterized by urinary concentrating defects such as nephrogenic diabetes insipidus (NDI), postobstructive polyuria, and acute and chronic renal failure (CRF), the apical expression of AQP2 is drastically reduced or even absent.

Urinary concentrating defects and polyuria are the most important renal manifestations of hypercalcemia and the resulting hypercalciuria. The mechanisms underlying the external calcium dependency of hypercalciuria-associated polyuria and subsequent alteration in AQP2 levels at the apical membrane are unknown but it is known that chronic hypercalcemia is associated with reduced NaCl reabsorption in the thick ascending limb. An increase in plasma Ca^{2+} concentrations stimulate the basolateral calcium-sensing receptor (CaR) in the medullary thick ascending limb and inhibit the apical K^+ conductance essential for maintaining the function of Na/K/Cl cotransporter [5]. Therefore, hypercalcemia causes a decrease in sodium reabsorption in the medullary thick ascending limb providing an explanation for the defect in diluting capacity [6]. Besides these effects, reduced concentrating ability in the collecting duct can contribute to the overall water reabsorption defect observed in hypercalciuria.

[1]Dr. Procino and Dr. Carmosino contributed equally to this work and must both be considered as first authors.

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We recently demonstrated that in primary nocturnal enuresis, a frequent disease in children, characterized by nocturnal polyuria, hypercalciuria was associated with alterations of urinary AQP2 levels indicating clearly that $Ca^{2+}{}_o$ activity modulates AQP2 excretion in the urine [7]. A relatively useful therapeutic intervention based on a low calcium diet demonstrated that amelioration of clinical is accompanied by regulation of urine output through remodulation of AQP2 expression/trafficking [8].

The close relation between hypercalciuria and AQP2 dysregulation was previously suggested by Sands et al [9] and, more recently, by Puliyanda et al [10]. These authors reported that, in rats, dihydrotachysterol (DHT) induces hypercalcemia/hypercalciuria associated with polyuria and AQP2 water channel down-regulation. A calcium-dependent calpain activation was proposed to modulate AQP2 levels through AQP2 proteolysis [10]. The kidney is a key organ for calcium homeostasis, and its ability to sense extracellular calcium levels in the urinary filtrate and the interstitial fluid is due to the extracellular CaR, which is expressed in multiple sites along the nephron [11, 12]. The renal CaR plays a crucial role in the regulation of divalent mineral cation transport [11, 13–15].

Inherited human diseases due to CaR gene mutations demonstrate the relevance of CaR in divalent mineral ion as well as water reabsorption. Heterozygous mutations causing CaR loss of function resulting in a lower sensitivity of the CaR to extracellular calcium such as in the familial hypocalciuric hypercalcaemia (FHH).

In this condition the kidney excretes less calcium causing relative hypocalciuria [16–18]. In contrast, CaR gain of function mutations like in autosomal-dominant hypocalcemia (ADH) lead to hypercalciuria [19]. CaR mutations have been demonstrated to alter renal water handling, supporting a role for the CaR in integrating mineral ion and water metabolism [20, 21]. Renal CaRs are expressed on the apical membrane of proximal tubule and on the basolateral membrane of thick ascending limb and in a diffuse pattern in the distal convoluted tubule [22]. In the collecting duct, CaRs are expressed at the apical membrane [23] where they might sense urinary calcium levels and influence AQP2 trafficking, providing a link between calcium and water homeostasis.

This work was undertaken to clarify the signal transduction pathways linking urinary calcium levels to AQP2 expression/trafficking in the collecting duct. The possible functional involvement of CaR in these events was also evaluated.

METHODS

Cell culture

CD8 cells were established by stably transfecting the RC.SV3 rabbit cortical collecting duct cells with the cDNA encoding rat AQP2 [24].

CD8 cells were grown at 37°C under a humidified atmosphere of 5% CO_2-95% air in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 1:1 (vol/vol) medium supplemented with 20 mmol/L sodium bicarbonate, 20 mmol/L Hepes, and 5% newborn calf serum.

Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of CaR cDNA from CD8 cells

Total RNA was extracted from confluent CD8 cells by the TRIzol extraction method (TRIzol Reagent) (Life Technologies, Gaithersburg, MD, USA). The RNA was then used to amplify fragments of cDNA from the CaR by degenerate RT-PCR. The degenerate primers CaR-1U (5′-ATCTGGCTGGCYAGCGAGGC-3′) and CaR-2D (5′-GCAATGGAGTAGACKGCTAAG-3′) were used. The oligonucleotides were designed based on the CaR nucleotide sequences from rat, human, and mouse.

A positive control was performed by using primers specific for β-actin cDNA (5′-CAGATCATGTTTGA GACCTT-3′) and (5′-CGGATGTCMACGTCACACA CTT-3′). The CaR cDNA amplified from the CD8 cells was cloned into the *Eco*RI/*Eco*RI site of the pCR2.1 vector (TA Cloning Kit; Invitrogen, San Diego, CA, USA) following a TA strategy. Cloned DNA fragments were controlled by sequencing.

Transfection of CD8 cells with human CaR-green fluorescent protein (GFP)

CaR sequence was generated by excising the 1373 bp *Eco*RI fragment from the human CaR sequence and cloning it into pcDNA3.1. A vector expressing a carboxy-terminal GFP-tagged human CaR was generated as previuosly described [25].

Transfection was performed by use of lipofectin. Cells were plated into 45 mm-dishes for 15 hours before transfection. Cells were washed with serum-free medium and then incubated for 12 hours at 37°C with 2 mL of serum-free medium containing 20 μg of lipofectin and 5 μg of recombinant plasmid. Cells were washed and then grown for 48 hours in DMEM supplemented with 5% fetal calf serum (FCS). Cells were trypsinized and plated on 140 mm diameter dishes. A selection of cells containing transfected DNA was obtained with a medium containing Hygromicin (500 μg/mL) for 10 to 15 days. Resistant clones were isolated and transferred to separate culture dishes for expansion and analysis.

Western blotting

For CaR detection by Western blotting in CD8 cells and in rat kidney papilla a technical procedure for intramembrane protein extraction was followed. Confluent monolayer of CD8 cells were rapidly scraped, pelleted in a microfuge, and homogenized by repeated freeze-thawing cycles (liquid nitrogen/37°C). The obtained homogenate

was extracted for 5 minutes in a solubilization buffer containing 150 mmol/L NaCl, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 $MgCl_2$ 10 mmol/L Tris, pH 7.4, 1% Triton X-114 in presence of 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL leupeptin, and 1 mg/mL pepstatin A at 4°C. Samples was then centrifuged at 14,000 × *g* for 15 minutes at 4°C to separate detergent-soluble fraction and detergent-insoluble fraction. The detergent insoluble fraction was completely solubilized in 1% NP-40 and 0.5% Triton X-100 in phosphate-buffered saline (PBS). The detetergent soluble fraction was used for Western blotting analysis (see below). For CaR detection in rat kidney, 500 μg of purified endosomes were extracted in 100 μL of the solubilization buffer followed by the same procedure employed for CD8 cells. AQP2 containing endosomes from rat papillae were prepared as described [26].

Equal amount of the proteins were resuspended in Laemmli buffer, heated to 60°C for 10 minutes and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA), blocked in blotting buffer (in mmol/L): 150 NaCl, 20 Tris-HCl, pH 7.4, and 1% Triton X-100, containing 5% nonfat dry milk, for 1 hour. The membranes were incubated with antiserum to CaR (Alexis Biochemicals, San Diego, CA, USA) (1:500) overnight at 4°C in blotting buffer, washed in several changes of the same blotting buffer. Immunolabeling controls were performed using preabsorption of the immune serum with the immunizing peptide for 2 hours at RT (1000-fold excess). The membranes were incubated with goat antirabbit IgG peroxidase-conjugated (Sigma-Aldrich Corporation, St. Louis, MO, USA) (1:5000 dilution) in the blotting buffer and visualized using the ECL-PLUS detection system (Bio-Rad, Hercules, CA, USA).

cAMP measurements

Intracellular cAMP accumulation was measured in CD8 cells cultured in 24-well plates to confluence (700,000 cells/wells). Cells were left under control conditions or were treated with 5 mmol/L calcium for 15 minutes or with 300 μmol/L Gd^{3+} for 15 minutes at 37°C in PBS-Ca^{2+}/Mg^{2+} (1 mmol/L Ca^{2+} and 1 mmol/L Mg^{2+}) and 100 μmol/L IBMX. Cells were then stimulated with 10^{-4} mol/L forskolin for 15 minutes at 37°C. The reaction was stopped by cooling the 24-well plates in ice. cAMP concentrations were measured in pmol per 10^6 cells and reported as a percentage of control cells, using a cAMP enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) as previously described [27].

Intracellular calcium measurements

Alterations of intracellular calcium concentration induced by a nonmembrane permeable CaR agonist, Gd^{3+}, were determined by a spectrofluorometric technique. CD8 cells were grown to confluence on glass coverslips. Cells were loaded with 10 μmol/L Fura-2/AM for 30 minutes in serum-free culture medium at 37°C. Cells were then washed once with a Fura-2-free bath solution (in mmol/L): 135 NaCl, 5.5 KCl, 1 $MgCl_2$, 1.26 $CaCl_2$, 0.44 KH_2PO_4, 0.64 Na_2HPO_4, 3 $NaHCO_3$, 5.5 glucose, and 20 Hepes, pH = 7.4. The coverslips were inserted into a specially designed cuvette as previously described [28]. The cuvette was connected to a perfusion system for solution changes, and fluorescence was recorded on a Shimadzu RF-5001 spectrofluorometer. The cells were alternatively illuminated with 340 and 380 nm light, and fluorescence at emission wavelength 510 nm was recorded. The cells were exposed to increasing $Ca^{2+}{}_o$ concentrations or to 300 μmol/L Gd^{3+} in the bath solution, and intracellular calcium concentration was calculated from the emission fluorescence ratio of the two excitation wavelengths using the formula: $[Ca^{2+}]i = K_d\,(R - R_{min})/(R_{max} - R)$ where Kd (dissociation constant) of Fura-2 for $[Ca^{2+}]_i$ was 224 nmol/L. Each sample was calibrated by the addition of 50 μmol/L digitonin (R_{max}) followed by 10 mmol/L ethyleneglycoltetraacetate (EGTA)/Tris (R_{min}).

Actin polymerization assay

The amount of F-actin was determined as described previously [29–31]. Briefly, CD8 cells were seeded on microplates 2 days before the experiments. Cells were left untreated (PBS-Ca^{2+}/Mg^{2+}) or stimulated with 10^{-4} mol/L forskolin for 15 minutes, or preincubated with 5 mmol/L calcium for 15 minutes or with 300 μmol/L Gd^{3+} for 15 minutes in PBS-Ca^{2+}/Mg^{2+}. The treatments were stopped by addition of a solution containing 3.7% formaldehyde, 0.1% Triton X-100, 0.25 μmol/L TRITC-phalloidin in 20 mmol/L potassium phosphate, 10 mmol/L PIPES, 5 mmol/L EGTA, and 2 mmol/L $MgCl_2$, pH = 6.8.

After staining for 1 hour, cells were washed with PBS and extracted with 800 mL cold methanol. The fluorescence (540/565 nm) was read in an RF-5301PC fluorometer. The values obtained were then analyzed by the Student *t*-test.

Quantitation of AQP2 cell surface immunoreactivity

CD8 cells were either left under control conditions (PBS-Ca^{2+}/Mg^{2+}), or stimulated with 10^{-4} mol/L forskolin for 15 minutes at 37°C in PBS-Ca^{2+}/Mg^{2+} in the presence or in the absence of 300 μmol/L Gd^{3+} or 5 mmol/L calcium. Cells were fixed and incubated with an antibody recognizing the extracellular AQP2 C loop (1:300 dilution in PBS-gelatin) for $1^1/_2$ hour at 4°C. Quantitation of cell surface immunoreactivity was performed as described [32].

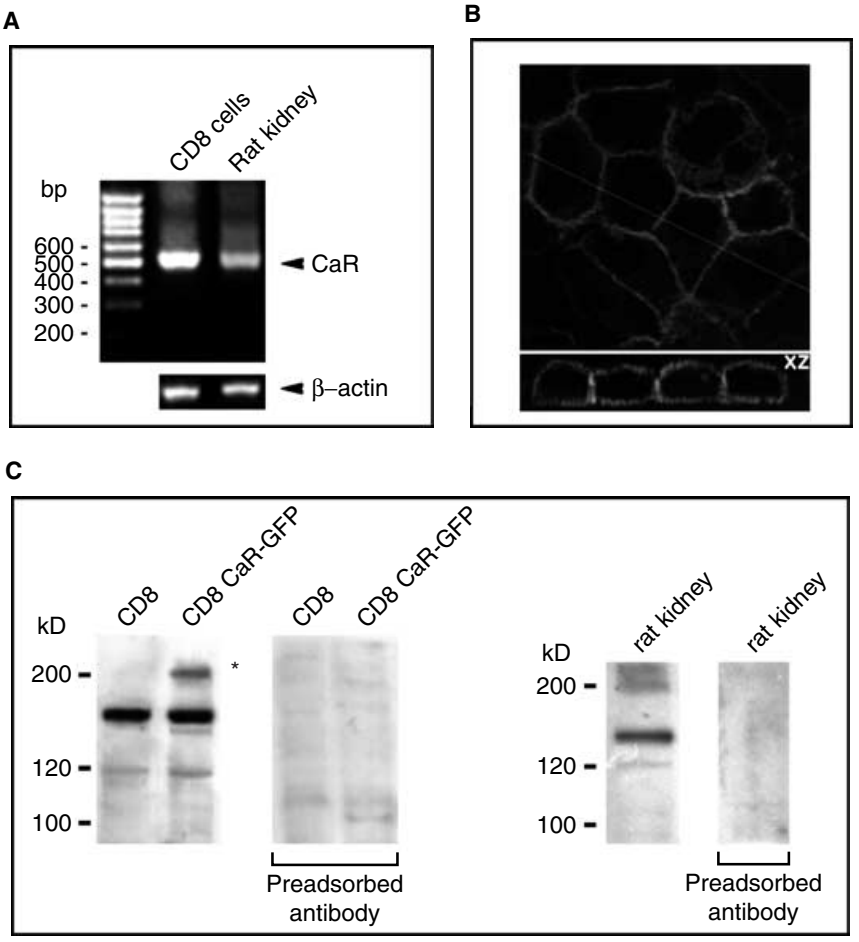

Fig. 1. Expression of calcium-sensing receptors (CaR) in CD8 cells. (*A*) Reverse transcription-polymerase chain reaction (RT-PCR) amplification of CaR cDNAs expressed in CD8 cells. Total RNA from confluent CD8 cells was subjected to reverse transcription followed by PCR amplification using degenerate primers for the coding region of known CaR (see **Methods** section for experimental details). Bands of 410 bp corresponding to CaR were amplified. As a positive control, parallel RT-PCR experiments with total RNA extracted from rat kidney were performed. Results shown are representative of at least three separate experiments. (*B*) Confocal analysis and the xz reconstruction (inset) performed on CD8 cells transfected with gel-filtered platelets (GFP)-tagged CaR. (*C*) Immunoblotting of Triton X114 soluble fraction from CD8 cell, CD8 CaR-GFP transfected cells and rat kidney endosomes. Each lane was loaded with 60 μg proteins. Blotted proteins were probed with anti-CaR serum (1:500 dilution) (Alexis Biochem) and immunoreactive bands were revealed with chemiluminescence ECL-PLUS (Bio-Rad). The same samples were incubated with anti-CaR antibody, which has been reabsorbed with the immunizing peptide (preadsorbed antibody). Asterisk corresponds to the CaR-GFP glycosylated form. Results shown are representative of at least three separate experiments.

Translocation of protein kinase C (PKC)

CD8 cells homogenate, prepared as described above, were subjected to 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were blocked in blotting buffer (in mmol/L): 150 NaCl, 20 Tris-HCl, pH 7.4, and 1% Triton X-100, containing 5% nonfat dry milk for 1 hour and incubated with anti-PKC isozymes (α, β, γ, δ, ε, ι, λ, θ, and η) monoclonal antibody (PKC Sampler Kit) (Transduction Laboratories), overnight at 4°C in blotting buffer. The membranes were incubated with goat antimouse IgG alkaline phosphatase conjugated (Sigma Chemical Co.) (1:5000 dilution) and revealed with 1-Step(tm) NBT/BCIP (Pierce, Rockford, IL, USA).

To study the membrane/cytosol expression of PKC, subconfluent monolayers of CD8 cells grown on 150 cm^2 culture Petri dishes were left under basal condition (PBS-Ca^{2+}/Mg^{2+}) or treated with 5 mmol/L $Ca^{2+}{}_o$ or with 300 μmol/L Gd^{3+} in PBS-Ca^{2+}/Mg^{2+} and then homogenized in ice-cold homogenization buffer [10 mmol/L Tris-HCl (pH 7.4), 0.25 mol/L sucrose, 2 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 5 mmol/L MgCl, 1 mmol/L PMSF, and a protease inhibitor cocktail (Sigma Chemical Co.)] and centrifuged at 100,000 × *g* for 1 hour at 4°C (Optima XL-100K ultracentrifuge) (Beckman Instruments, Palo Alto, CA, USA). The resulting pellets and supernatants were collected and referred to as membrane and cytosolic fractions, respectively. Membranes or cytosol fractions were solubilized in Laemmli buffer, heated to 60°C for 10 minutes, and subjected to 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were probed with anti-PKC isozymes antibodies as described above.

Data analysis and statistics

Data are expressed as mean ± SE. Statistical analysis was performed by unpaired Student *t* test or by one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons. Band intensities in Western blotting analysis were quantitated by densitometric analysis using National Institutes of Health Images (NIH) software.

RESULTS

CD8 cells express functional endogenous CaR

To test the expression of CaR in CD8 cells, RT-PCR studies were carried out. The RT-PCR experiments were performed with total RNA extracted from confluent CD8

cells using degenerated primers, based on conserved regions of CaR nucleotide sequences (see the **Methods** section). As shown in Figure 1A, a fragment having an expected size of 410 bp was amplified both from rat kidney and CD8 cell total RNA. The specificity of the cDNAs amplified by RT-PCR was confirmed by cloning and sequencing of the PCR fragment. The fragment was found to share 91% identity with the homosapiens CaR mRNA.

In the kidney collecting duct, CaR has been localized to the apical membrane of principal cells [23]. To analyze the localization of the CaR, CD8 cells were stably transfected with the human CaR-GFP. This was necessary because of the lack of immunoreactivity of the available antibody in immunolocalization studies. Confocal analysis and the xz reconstruction showed that GFP-tagged CaR was localized both to the basolateral and apical membrane domain with an apparent more pronounced lateral localization (Fig. 1B). A very low intracellular staining was observed.

To analyze the expression of the CaR as proteins, Western blot experiments were carried out using specific antibodies. CD8 cells homogenate or endosomes from rat kidney papillae were treated with Triton X-114 as described in the **Methods** section. Figure 1C shows the Western blotting of the Triton X-114 soluble fraction from CD8, CaR-GFP-transfected CD8 cells and rat kidney probed with anti-CaR antibodies. A 120 kD band corresponding to the monomeric nonglycosylated form of CaR as well as a stronger 160 to 170 kD band corresponding to the mature glycosylated form in both CD8 cell and CaR-GFP-transfected CD8 cells were stained. As expected, in CaR-GFP transfected cells, besides the endogenous CaR immureactive bands, an additional band around 200 kD (about 30 kD greater than the size of the endogenous mature receptor) (Fig. 1C, asterisk) was detected corresponding to the GFP-tagged CaR. Moreover, a faint band around 150 kD was also stained, likely corresponding to the monomeric nonglycosylated GFP-tagged CaR.

In the Triton-X114 soluble fraction of endosomes from rat papilla the antibody recognized the conventional 120 kD band and a stronger140 kD band corresponding to the glycosylated form of CaR. An additional band was visible over 200 kD probably corresponding to the dimeric form of CaR. Compared with CD8 cells, the glycosylated form of the CaR showed a slightly lower molecular weight probably due to the different glycosylation pattern of the protein in rabbit and rat, respectively.

The binding of the anti-CaR polyclonal antibody to the CaR bands was shown to be specific because it was ablated by preincubation of antibody with excess immunizing peptide (preadsorbed antibody).

To determine whether the CaR in CD8 cells is a functional receptor, we tested alteration in intracellular Ca^{2+} concentrations in response to increasing $Ca^{2+}{}_o$ or to the CaR agonist Gd^{3+}. Raising $Ca^{2+}{}_o$ from 0.2 mmol/L to 2.5 or to 5 mmol/L caused a dose-dependent elevation in $[Ca^{2+}]_i$ from 67.5 ± 9.1 nmol/L to 120 ± 12 nmol/L or to 305 ± 20.5 nmol/L, respectively (Fig. 2A and A′). When CD8 cells were perfused with 300 μmol/L Gd^{3+}, $[Ca^{2+}]_i$ concentration increased by about 1.5-fold over basal intracellular Ca^{2+} levels (from 80 ± 6.3 nmol/L to 110.4 ± 6.7 nmol/L) (Fig. 2B and B′). Similar results were obtained in the absence of $Ca^{2+}{}_o$, confirming that CaR stimulation induces the calcium release from the intracellular stores (Fig. 2B, inset).

Extracellular calcium impairs forskolin-induced cell surface expression of AQP2

AQP2 targeting in response to forskolin was quantified in the presence or in the absence of 5 mmol/L $Ca^{2+}{}_o$ or in the presence of 300 μmol/L Gd^{3+}. To this end, cell surface immunoreactivity of anti-AQP2 antibody raised against a peptide reproducing the extracellular AQP2 C loop was evaluated under different experimental conditions (Fig. 3). As expected, in the presence of physiologic extracellular calcium concentrations, forskolin increased cell surface expression of AQP2 by about twofold, as a consequence of the increase in intracellular cAMP levels as shown previously (Fig. 3) [32]. In contrast, in the presence of 5 mmol/L $Ca^{2+}{}_o$ or Gd^{3+}, forskolin did not significantly increase the cell surface abundance of AQP2. These results demonstrate an antagonistic effect of extracellular calcium on forskolin-stimulated AQP2 trafficking, suggesting that CaR plays a modulatory role in vasopressin-induced antidiuresis.

cAMP levels are drastically reduced in forskolin-stimulated cells in the presence of $Ca^{2+}{}_o$ and GD^{3+}

We next investigated the intracellular signals downstream of extracellular calcium and Gd^{3+} stimulation and leading to a reduction of AQP2 targeting upon forskolin stimulation. As $[Ca^{2+}]_i$ regulates cAMP levels in many cell types through interaction of calcium on cAMP synthesis and/or hydrolysis [33, 34], we first analyzed whether a forskolin-elicited increase in cAMP levels is influenced by $Ca^{2+}{}_o$. When CD8 cells were stimulated with forskolin (10^{-4} mol/L, 15 minutes), cAMP levels increased by 14.79 ± 1.6-fold ($N = 18$) with respect to control cells (Fig. 4). In contrast, when forskolin stimulation was performed in the presence of 5 mmol/L $Ca^{2+}{}_o$, a drastic reduction in the rise of cAMP levels was measured, corresponding to 4.40 ± 1.0-fold ($N = 6$) and 4.0 ± 0.3-fold ($N = 10$), respectively (Fig. 4). cAMP levels in response to forskolin stimulation were also measured in the presence of 300 μmol/L Gd^{3+}, a known CaR activator. Interestingly, in the presence of Gd^{3+}, forskolin stimulation reduced the cAMP concentration by about 50% (from 14.79 ± 1.6-fold to 7.45 ± 1.62-fold, $N = 10$) (Fig. 4).

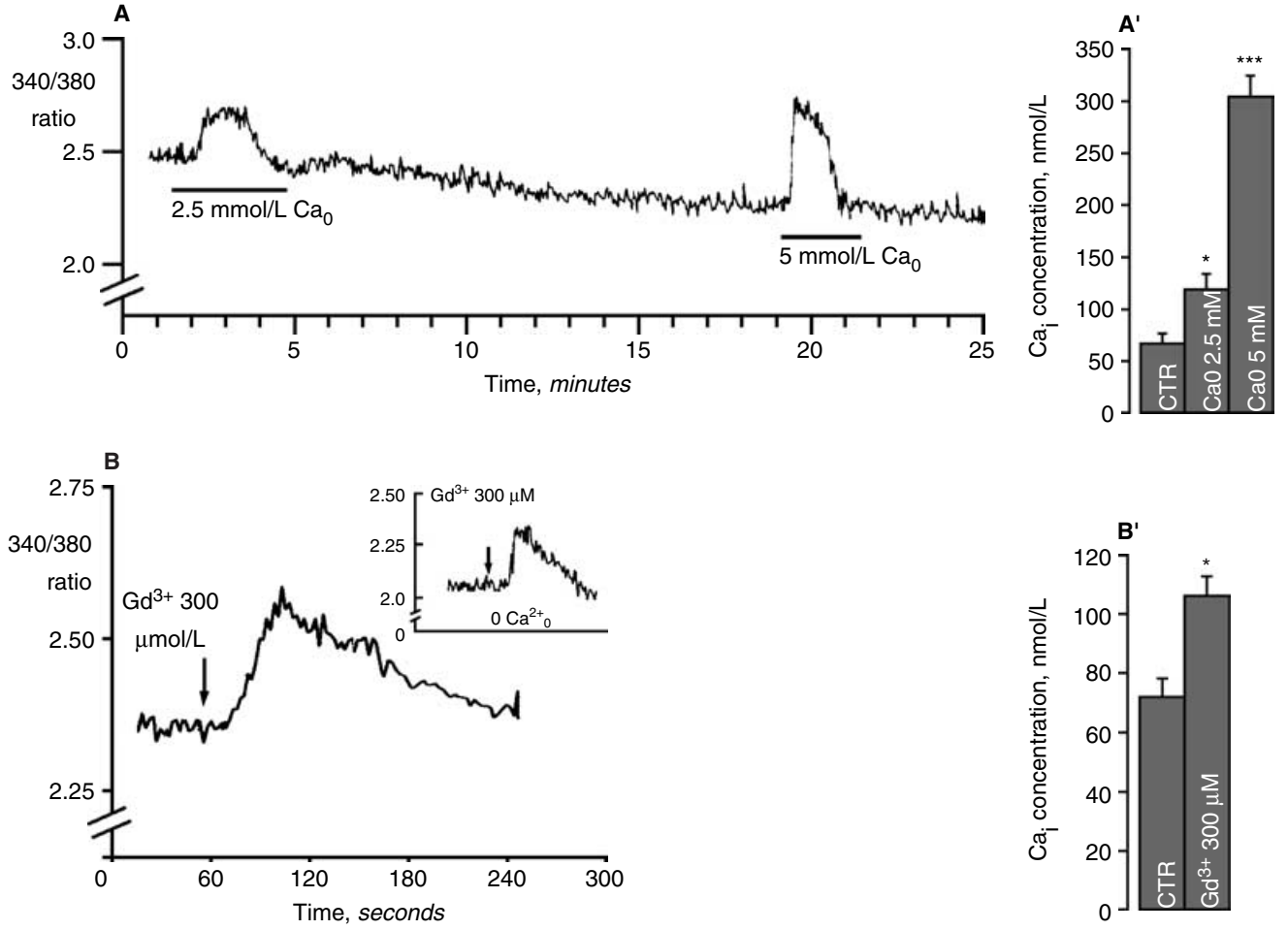

Fig. 2. Effect of Ca^{2+}_o and Gd^{3+} on $[Ca^{2+}]_i$ concentration in CD8 cells. (*A*) Representative trace of the time course of the emission ratio (340/380 excitation) in Fura-2–loaded CD8 cells perfused with increasing concentration of extracellular calcium. (*A'*) Mean ± SE of $[Ca^{2+}]_i$ concentrations in basal condition, after 2.5 mmol/L Ca^{2+} and after 5 mmol/L Ca^{2+} ($N = 3$). $^*P < 0.05$; $^{***}P < 0.001$ vs. values calculated in basal condition by one-way analysis of variance (ANOVA and Tukey's multiple comparison test). (*B*) Representative experiment showing the effect of 300 µmol/L Gd^{3+} over 300 seconds. Similar results were obtained in the absence of Ca^{2+}_o, confirming that calcium-sensing receptor (CaR) stimulation induces the calcium release from the intracellular stores (inset). (*B'*) Mean ± SE of $[Ca^{2+}]_i$ concentrations in control and Gd^{3+}-treated cells ($N = 3$). $P < 0.05$ vs. values calculated in control cells by Student *t* test.

Extracellular calcium causes PKC activation

In various cell types, elevation of extracellular calcium elicits a complex signaling cascade leading to phospholipase C (PLC)-dependent stimulation of PKC [35]. Since it has been suggested that PKC activation may mediate endocytosis of AQP2 in renal cells [36], we tested whether CaR activators, Ca^{2+}_o and Gd^{3+}, could cause PKC activation in CD8 cells. We first determined the expression of PKC isoforms in CD8 cells by Western blotting using isoform-specific antibodies.

In cell homogenates from CD8, five different isoforms of PKC (α, δ, ε, ι, and λ) were found to be expressed, while PKC isoforms β, γ, θ, and η were not detectable (Fig. 5A). Activation of PKC isoforms was assessed by their ability to translocate from cytosol to membrane as a consequence of CaR signaling cascade (Fig. 5B, B′, C, and C′). Phorbol 12-myristate 13-acetate (PMA) was used as positive control. Under basal condition, 50% PKCα and 26.5% PKCε were expressed in the membrane fraction. Exposure to 5 mmol/L Ca^{2+}_o or 300 µmol/L Gd^{3+}caused an enrichment of both PKCα and ε in the membrane fraction (PKCα from 50 ± 3.7% to 76.5 ± 5.6%; from 50 ± 3.7% to 74.5 ± 4%, respectively; PKCε from 26.5 ± 4.9% to 58.3 ± 3.2% and from 26.5 ± 4.9% to 62 ± 0.5%, respectively). A concomitant proportional decrease of PKCα and PKCε in the cytosol fraction was observed. Neither Ca^{2+}_o nor Gd^{3+} had any effect on the cellular localization of PKC isoforms δ, ι, and λ (data not shown).

Ca^{2+}_o and GD^{3+} induce formation of F-actin-containing stress fibers

In human embryonic kidney (HEK)-293 cells transfected with CaR, a CaR-mediated increase in membrane-associated RhoA has been demonstrated, suggesting a

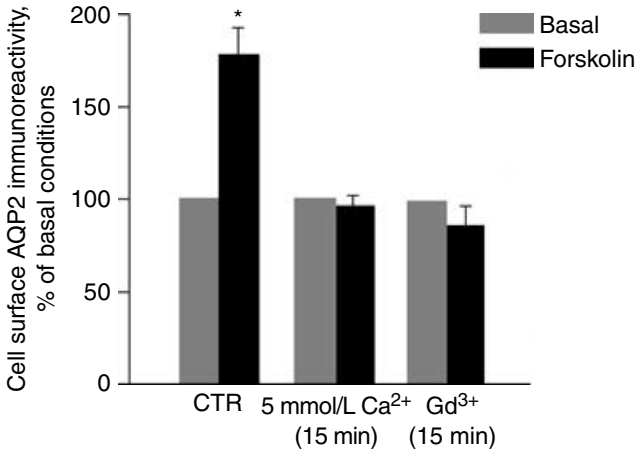

Fig. 3. Determination of cell surface aquaporin 2 (AQP2) immunoreactivity. The anti-AQP2 C loop antibody was employed to monitor the AQP2 abundance on the plasma membrane in CD8 cells as previously described [28]. In untreated cells, forskolin stimulation increased the immunodetectable AQP2 on the cell surface by approximately twofold. In contrast, when forskolin stimulation was performed in the presence of 5 mmol/L $Ca^{2+}{}_o$ or in the presence of 300 μmol/L Gd^{3+}, this abolished the forskolin-induced increase in cell surface expression of AQP2. Results shown represent the means ± SE of three separate experiments in which about 4×10^6 cells (six separate wells) were tested for each experimental condition in each experiment. Results are expressed as percent of the value calculated under the respective basal conditions. $^{**}P < 0.01$ vs. values calculated in control cells by Student *t* test.

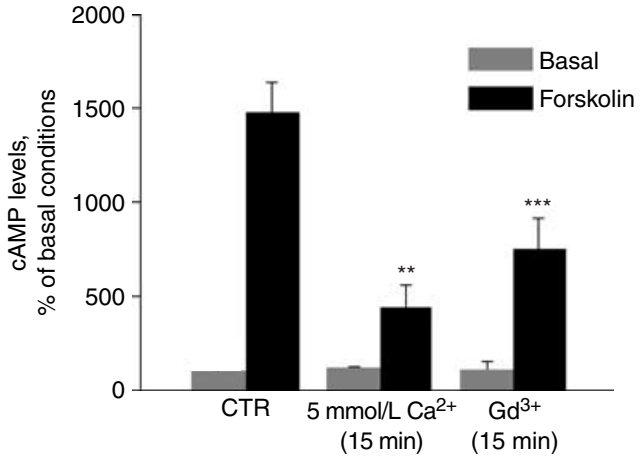

Fig. 4. Measurements of intracellular cyclic adenosine monophosphate (cAMP) concentration in response to forskolin stimulation. CD8 cells were stimulated with forskolin (10^{-4} mol/L at 37°C for 15 minutes) in the absence or in the presence of 5 mmol/L $Ca^{2+}{}_o$ or of 300 μmol/L of the calcium-sensing receptor (CaR) agonist Gd^{3+}. Results are expressed as percent of the value calculated under the respective basal conditions. $P < 0.01$; $^{***}P < 0.001$ vs. values calculated in forskolin-stimulated cells under control condition by Student *t* test.

CaR activation of Rho [37]. On the other hand, earlier work from our laboratory demonstrated that in CD8 cells, stabilization of actin cytoskeleton obtained by expression of constitutive active Rho leads to formation of F-actin-containing stress fibers and impairs AQP2 trafficking upon forskolin exposure [38, 39].

We therefore evaluated whether treatment with CaR agonists results in any alteration of actin assembly/disassembly. The results of the quantitative actin polymerization assay are reported in Figure 6. Interestingly, with respect to control cells, the F-actin content increased dramatically after exposure to 5 mmol/L calcium or after treatment with Gd^{3+} (from 79.2 ± 5.1 to 147.3 ± 14.4 and 111.7 ± 15.40, respectively). In contrast, forskolin stimulation resulted in a significant (25%) decrease in F-actin content.

Visualization of F-actin by phalloidin-TRITC was in full agreement with the quantitative data obtained with the actin polymerization assay (Fig. 7).

DISCUSSION

While it has been suggested that the luminal calcium plays an important role in hypercalciuria-induced polyuria, the molecular basis of this effect has not been well established.

Previous studies have demonstrated the presence of CaR protein in the apical membrane of rat collecting duct [23]. In isolated perfused rat inner medullary collecting duct, acute increases in luminal calcium from 1 to 5 mmol/L calcium caused a rapid 30% reduction in vasopressin-elicited P_f [24]. In addition, during sustained hypercalcemia in chronically hypercalcemic rats, the P_f in inner medullary collecting duct did not increase significantly after vasopressin and was accompanied by an 87% reduction in AQP2 protein [9], which may contribute to the lack of vasopressin responsiveness. Two previous observations of ours prompted us to investigate the possible mechanism(s) by which extracellular signaling might modulate renal water handling through alteration of AQP2 trafficking/expression. In the first, we have shown that hypercalciuria is associated with alterations of urinary AQP2 levels in enuretic children [7]. Second, we demonstrated that lowering urinary calcium through a low calcium diet normalized urinary AQP2 excretion and improved the clinical symptoms [8]. Those observations imply that an apical membrane signaling mechanism links calcium and water permeability in the renal collecting duct. In this study, we were therefore interested in identifying the molecular mechanisms that link hypercalciuria-associated polyuria and AQP2. The present contribution demonstrates a clear regulatory role of extracellular calcium on AQP2 trafficking in renal collecting duct cells.

To gain insights into a potential role for the CaR in the regulation of AQP2 trafficking, we used AQP2-transfected collecting duct CD8 cells [24]. This cell line expresses endogenous CaR as assessed by RT-PCR and sequencing using nested CaR primers. We report here that, in CD8 cells, CaR activation caused an increase in $[Ca^{2+}]_i$, as a result of calcium release from intracellular stores. Interestingly, both $Ca^{2+}{}_o$ and Gd^{3+} drastically

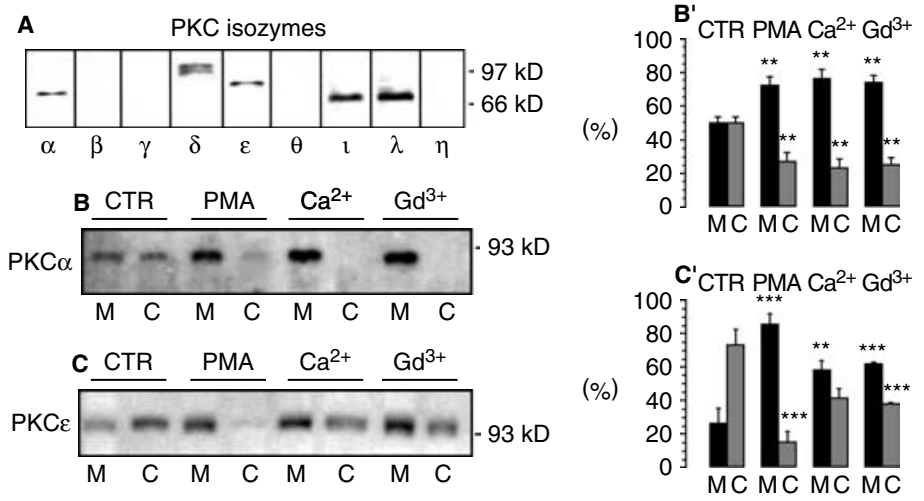

Fig. 5. Extracellular calcium and Gd^{3+} induce protein kinase C (PKC) translocation. (*A*) Immunoblots of PKCα, β, γ, δ, ε, θ, ι, λ, and η in CD8 cells homogenate. Results are representative of four independent experiments. (*B* and *C*) Translocation of PKCα and PKCε induced by calcium-sensing receptor (CaR) agonists $Ca^{2+}{}_o$ and Gd^{3+}. Results are representative of four experiments showing that PKCα and PKCε translocated from the cytosol to the membrane fraction. (*B*' and *C*'). Statistical analysis refers to relative PKC abundance in membrane and cytosol fractions upon stimulation with $Ca^{2+}{}_o$ and Gd^{3+}. Results are expressed as mean ± SE. Abbreviations are: C, cytosol; M, membrane ($N = 4$). $^{**}P < 0.01$; $^{***}P < 0.001$ vs. values calculated for the same subcellular fraction under control condition by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

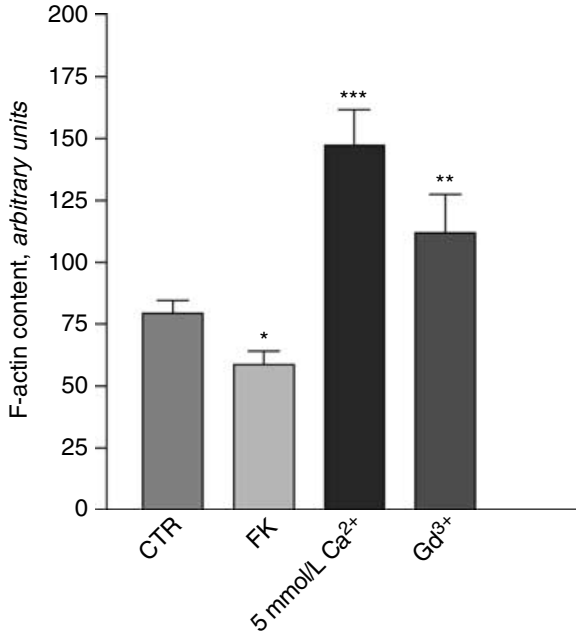

Fig. 6. Actin polymerization assay. CD8 cells were left untreated or stimulated with forskolin 10^{-4} mol/L for 15 minutes. Alternatively, cells were preincubated with 5 mmol/L calcium for 15 minutes and with Gd^{3+} 300 μmol/L for 15 minutes. To quantify F-actin content, cells were then incubated in a solution containing TRITC-phalloidin and extracted with methanol (see the **Methods** section for details). The fluorescence (540/565 nm) was read and the values compared by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

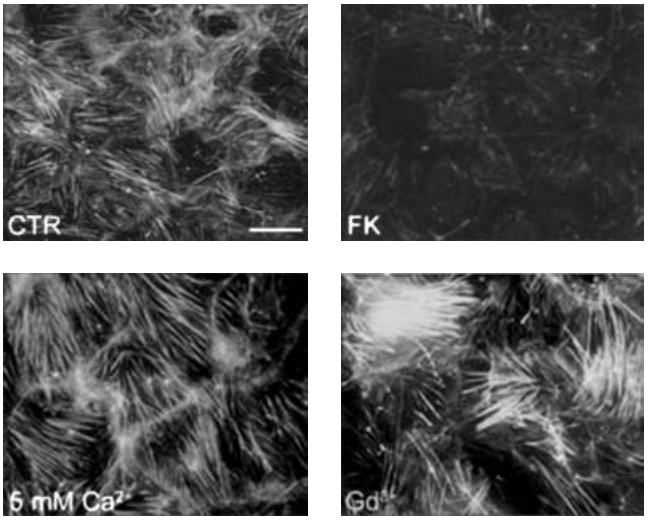

Fig. 7. Fluorescence visualization of F-actin in CD8 cells. Cells were left untreated (CTR) or incubated with 5 mmol/L $Ca^{2+}{}_o$ or with 300 μmol/L Gd^{3+} for 15 minutes at 37°C. Cells were then fixed and F-actin was stained with TRITC-conjugated phalloidin. Fluorescence was detected by epifluorescence microscopy. Results shown are representative of at least three separate experiments (bar = 10 μm).

(~70%) inhibited forskolin-induced cAMP accumulation. Consistent with that, we demonstrated that either Gd^{3+} or 5 mmol/L $Ca^{2+}{}_o$ strongly inhibited the forskolin-stimulated increase in AQP2 immunoreactivity on the apical plasma membrane, as assessed by AQP2 immunoquantification. These data demonstrate that CaR agonists evoke an inhibition of forskolin-induced cAMP accumulation and, in turn, this may be in part responsible for the impaired AQP2 targeting to the plasma membrane. Based on our in vitro results we can exclude that both $Ca^{2+}{}_o$ and Gd^{3+} confer inhibition of cAMP accumulation through direct inhibitory protein G (Gi)-mediated inhibition of adenylate cyclase, as this effect is measurable upon direct activation of the catalytic subunit of

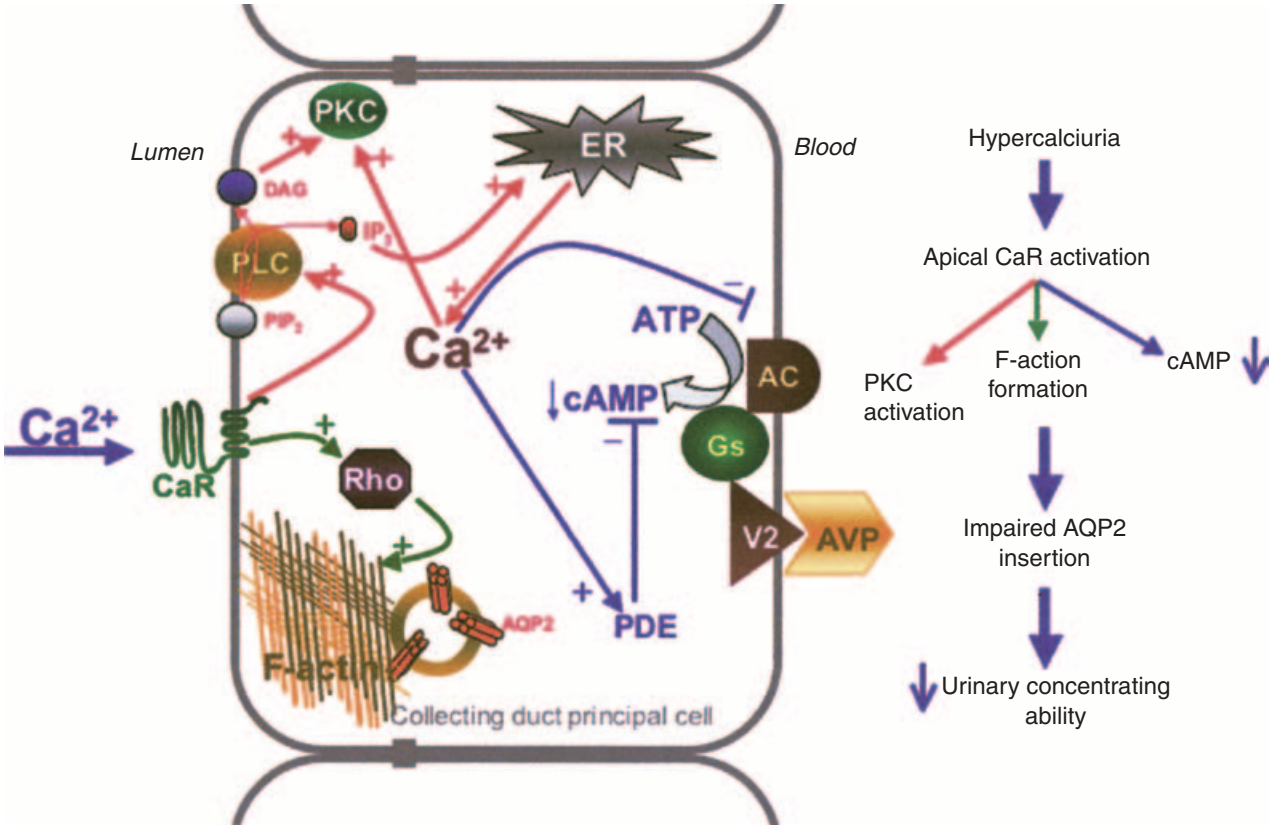

Fig. 8. Proposed mechanism by which $Ca^{2+}{}_o$, possibly acting through the calcium-sensing receptor (CaR), interferes with water reabsorption in inner medullary collecting duct. Extracellular calcium activates the CaR, leading to increases in $[Ca^{2+}]_i$ possibly via phospholipase C (PLC) activation. This decreases forskolin-induced cyclic adenosine monophosphate (cAMP) levels through inhibition of adenyl cyclase and/or activation of phosphodiesterase. In addition, $[Ca^{2+}]_i$ and diacylglycerol (DAG) can activate calcium-dependent and calcium-independent protein kinase C (PKC) isoform. CaR activation also results in formation F-actin–containing stress fibers probably through Rho activation. All these processes contribute to reduction of forskolin-induced aquaporin 2 (AQP2) targeting to the plasma membrane thus decreasing water reabsorption from the lumen which would contribute to impairment of kidney concentrating ability. Abbreviations are: ER, endoplasmic reticulum; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; AC, adenylate cyclase; Gs, α subunit of the s-type heterotrimeric G proteins; V2, vasopressin type 2 receptor; AVP, arginine-vasopressin; PDE, phosphodiesterase.

adenylate cyclase by forskolin. However, while our in vitro data would support a post receptor effect of CaR activation in inhibiting AQP2 translocation, it is possible that, in vivo a Gi coupled pathway may also be involved. Vasopressin activates sodium and water reabsorption in the thick ascending limb and medullary collecting duct, respectively, by increasing cAMP levels. These effects are attenuated in the presence of high $Ca^{2+}{}_o$. The existing data, in rat tubules, based on the use of pertussis toxin, support that a Gi coupled receptor activation signaling may mediate the extracellular calcium effects [40–42]. In some cells expressing CaR, CaR agonists can inhibit cAMP accumulation through a mechanism involving a CaR-induced increase in $[Ca^{2+}]_i$ which then inhibits a calcium-sensitive form of adenylate cyclase [43]. This mechanism may explain the reduction in cAMP levels. Alternatively Ca^{2+}-dependent increase in $[Ca^{2+}]_i$ can impair cAMP accumulation by an increase in cAMP hydrolysis, through a Ca^{2+}-mediated process [33, 34].

We also show here that treatment with 300 μmol/L Gd^{3+} or with 5 mmol/L $Ca^{2+}{}_o$ for 15 minutes caused activation of PKCα and PKCε. The former belongs to the conventional PKC isoforms that require calcium and diacylglycerol (DAG) for activation while the latter belongs to the novel PKC isoforms that are calcium-independent but require DAG for activation. PKC can contribute to the inhibitory effect of $Ca^{2+}{}_o$ on forskolin-induced AQP2 targeting to the plasma membrane. Indeed, Van Balkom et al [36] suggested that PKC activation may mediate endocytosis of AQP2 in renal cells.

Another intriguing effect of $Ca^{2+}{}_o$ and Gd^{3+} in renal CD8 cells was the activation of F-actin assembly. We have recently demonstrated that cAMP-dependent AQP2 translocation is associated with inhibition of RhoA, which results in F-actin disassembly [25, 37, 43]. Conversely, we also have shown that the diuretic effect of prostaglandin E_2 (PGE_2) on renal principal cells is mediated by Rho activation resulting in F-actin assembly [44].

We report here that, while forskolin or vasopressin treatment induced a significant decrease in F-actin content (25%), 5 mmol/L calcium or Gd^{3+} treatment resulted

in a nearly twofold increase in F-actin content compared with basal conditions. Stabilization of cortical F-actin cytoskeleton may impair AQP2 targeting, as previously suggested [38, 44]. We may speculate that F-actin assembly is a result of Rho activation associated with CaR signaling. Indeed, in HEK-293 cells transfected with CaR, a CaR-mediated increase in membrane-associated RhoA has been demonstrated, suggesting a CaR activation of Rho [39]. Moreover, in HEK-293 cells it has been demonstrated that Gαq and Gαq-coupled receptors activate Rho and its downstream effectors [45, 46]. Interestingly, Gαq is a G protein to which the CaR can couple [11, 47] and AQP2-containing vesicles have been shown to possess Gαq [23].

Here, we speculate that F-actin assembly is a result of Rho activation possibly associated with CaR signaling.

CONCLUSION

The data reported here provide the first physiologic explanation for an effect of luminal calcium in kidney inner medulla. Specifically, we demonstrated that luminal calcium antagonizes AQP2 translocation to the apical plasma membrane through a reduction in cAMP levels, activation of PKC and stabilization of actin cytoskeleton (see proposed model in Fig. 8). Given that the observed effects could also be mediated by another CaR agonist, Gd^{3+}, our observations suggest a role for a CaR-mediated inhibition of the vasopressin response. Physiologically, "sensing" the increase in urinary calcium by CaR would reduce vasopressin-stimulated water reabsorption from urine to interstitial fluid. This response would prevent further calcium concentration, thus protecting against a potential risk of calcium oxalate- or calcium phosphate-containing kidney stone formation. Further work using specific CaR modulators is necessary to demonstrate unequivocally a role for the CaR in this phenomenon.

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Reprint requests to Dr. Giovanna Valenti, University of Bari, Dipartimento di Fisiologia Generale ed Ambientale, Via Amendola 165/A, 70126 Bari, Italy.
E-mail: g.valenti@biologia.uniba.it

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