Differential Modulation of Intracellular Ca^{2+} Responses Associated with Calcium-Sensing Receptor Activation in Renal Collecting Duct Cells

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Key Words

Calcium sensing receptor • Rho • NPS-R 568 • Kidney

Abstract

In this work, we studied G protein-coupled Extracellular Calcium Sensing Receptor (CaR) signaling in mouse cortical collecting duct cells (MCD4) expressing endogenous CaR. Intracellular $[\text{Ca}^{2+}]$ measurements performed with real time video imaging revealed that CaR stimulation with 5mM Ca^{2+} , 300 μM Gd^{3+} and with 10 μM of specific allosteric modulator NPS-R 568, all resulted in an increase in $[\text{Ca}^{2+}]_i$ although displaying different features. Specifically, Ca^{2+} as well as stimulation with NPS-R 568 induced a rapid peak of $[\text{Ca}^{2+}]_i$ while stimulation with Gd^{3+} induced transient intracellular Ca^{2+} oscillations. PLC inhibition completely abolished any $[\text{Ca}^{2+}]_i$ increase after stimulation with CaR agonists. Inhibition of Rho or Rho kinase (ROK) abolished $[\text{Ca}^{2+}]_i$ oscillations induced by Gd^{3+} , while the peak induced by high Ca^{2+} was similar to control. Conversely, emptying the intracellular calcium stores abolished the response to Gd^{3+} . On the other hand,

the inhibition of calcium influx did not alter calcium changes. We conclude that in our cell model, CaR stimulation with distinct agonists activates two distinct transduction pathways, both PLC-dependent. The transient cytosolic Ca^{2+} oscillations produced by Gd^{3+} are modulated by Rho-Rho kinase signaling, whereas the rapid peak of intracellular Ca^{2+} in response to 5mM $[\text{Ca}^{2+}]_o$ is mainly due to PLC/IP3 pathway activation.

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Introduction

The Extracellular Calcium Sensing Receptor (CaR) is a G-protein coupled receptor originally cloned from the bovine parathyroid gland [1] and successively identified in various organs [2, 3].

The CaR enables key tissues to sense extracellular calcium by detecting small changes in calcium levels. Increases in extracellular calcium cause activation of the

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receptor, which in turn directly or indirectly modulates various homeostatic responses in order to normalize calcium. Besides the parathyroid gland, the key CaR-expressing organs are intestine, bone, and kidney [4]. However, in the kidney, CaR has been found to be expressed in regions not known to play a role in Ca^{2+} metabolism [5]. Within the nephron segments, CaR protein is luminal in the proximal tubule and collecting duct and basolateral in the thick ascending limb of Henle's loop.

The apical localization of CaR in the collecting duct suggests that the receptor is activated by luminal calcium, raising the hypothesis that it plays a role in fine-tuning water reabsorption in response to vasopressin, as initially suggested by Sands and coworkers [6]. This hypothesis is supported *in vitro* in collecting duct cell lines, demonstrating that CaR agonist strongly inhibited forskolin-stimulated increases in cell surface expression of AQP2 in the plasma membrane [7]. Along the same lines, more recently it has been reported that in mpkCCD cells, high concentrations of extracellular calcium attenuate vasopressin-induced AQP2 expression by activating the CaR and reducing the efficiency of coupling between the V2 receptor and adenylate cyclase [8].

The cellular responses to CaR activation are first mediated by the specific activated G protein to which it is coupled. Upon calcium binding, CaR may activate G-proteins, $G_{q/11}$, G_i , and $G_{12/13}$, which stimulate phospholipase C (PLC) resulting in production of diacylglycerol and IP_3 , causing release of calcium from intracellular stores, inhibition of adenylate cyclase and activation of Rho kinase respectively [9]. Inhibition of adenylate cyclase causes a reduction in cAMP levels. In some cell types, it has been shown that the latter effect can also be the consequence of CaR-dependent activation of phosphodiesterase [10]. On the other hand, in a few cell types, CaR activates G_s , the stimulatory G protein stimulating adenylate cyclase [11]. CaR regulates many other intracellular pathways, including mitogen-activated protein kinases (MAPKs), phospholipases A_2 and D, and the epidermal growth factor [9].

To gain insights into the signal transduction activated by CaR stimulation in a specific nephron segment, in this work we studied the signal transduction pathways activated by CaR endogenously expressed in mouse cortical collecting duct cells (MCD4).

Interestingly, the data obtained indicate that CaR stimulation with distinct agonists promotes two distinct transduction pathways due to coupling to distinct G proteins and characterized by two different profiles in the increase in intracellular calcium.

Materials and Methods

Cell culture

Mouse cortical collecting duct MCD4 cells, stably expressing human AQP2 were generated as described elsewhere [12] and maintained in DMEM/F12 1:1 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 5 μM dexamethasone until sub confluent.

Solutions and Materials

All chemicals were purchased from Sigma. Fura-2 AM was obtained from Molecular Probes (Eugene, OR). C3 Transferase was purchased from Cytoskeleton. NPS-R568 and the enantiomer NPS-S 568 were a gift from Amgen. Experiments were performed with a Ringer solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl_2 , 1 CaCl_2 , 5 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (adjusted to pH 7.4 with NaOH). In experiments with high Ca^{2+} , the ion concentration was raised with CaCl_2 .

Antibodies

Affinity purified Anti rabbit antibodies against the C-terminus of CaR were purchased from Chemicon (Millipore Corporate Headquarters, Billerica, USA). Affinity purified Anti rabbit antibodies against $G_{\alpha q}$, $G_{\alpha 12}$, $G_{\alpha 13}$, and RhoA were purchased from Santa Cruz (Santa Cruz Biotechnology, California, USA).

Reverse transcription-Polymerase chain reaction (RT-PCR)

Total RNA was extracted from confluent MCD4 cells by the TRIzol extraction method (TRIzol Reagent®-Invitrogen, San Diego, CA, USA). The RNA was then used to amplify fragments of cDNA from the CaR by degenerate RT-PCR. The degenerate primers CaR Rev 5'TGC AAT TGT CCC ACC CAG T 3' and CaR Fw 5'ACC AGC GAG CCC AAA AGA AG3' were used. The oligonucleotides were designed based on the CaR nucleotide sequences straddle two different exons in the identity regions of rat, human and mouse. A positive control was performed by using primers specific for β -actin Rev 5'CTC TCA GCT GTG GTG GT3' and β -actin Fw 5'AGC CAT GTA CGT AGC CAT3' based on the Actin nucleotide sequences from rat, and primers specific for β -actin Rev 5'CTC TCA GCT GTG GTG GTG AA3' and β -actin Fw 5'AGC CAT GTA CGT AGC CAT CC3' based on the Actin nucleotide sequences from mus musculus.

Cell fractionation and cell homogenate

For plasma membranes preparations from MCD4, cells were resuspended in homogenizing buffer (300mM sucrose, 25mM imidazole, 1mM EDTA, 1mM PMSF, protease cocktail inhibitor, pH 7.2) and homogenized with 5 strokes in a Potter-Elvehjem at 1,250rpm. The homogenate was spun at 4,000xg for 15 min and the pellet discarded. The supernatant was spun at 17,000xg for 30 min to obtain a pellet enriched in plasma membranes. Fractions were assayed for protein content and stored at -80°C .

MCD4, and kidney homogenates were prepared in RIPA Buffer (150mM NaCl, 10mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5mM EDTA).

Gel electrophoresis and Immunoblotting

Cellular proteins (homogenates or subcellular fractions) were separated on 4–12% NuPAGE Bis-Tris gels under reducing conditions. Protein bands were electrophoretically transferred to ImmobilonP membranes (Millipore Corporate Headquarters, Billerica, USA) for Western blot analysis, blocked in TBS-Tween containing 3% BSA and incubated with primary antibodies. Immunoreactive bands were detected with secondary antibody conjugated to horseradish peroxidase (HRP). After each step, the membranes were washed with TBS-Tween. Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) and were exposed to autoradiographic Kodak Biomax XAR film, (Sigma-Aldrich, St. Louis, USA). Band intensities were quantified by densitometric analysis using National Institutes of Health (NIH) ImageJ software.

Intracellular Ca^{2+} measurements

For intracellular Ca^{2+} measurements, cells were grown on Ø40mm glass coverslips and used 2–3 days after seeding. Cells were dye loaded by incubation for 30 min at 37°C in culture medium containing 10µM Fura-2 AM. After dye loading, cells were washed three times with Ringer solution and the coverslip was mounted on a microperfusion chamber. $[Ca^{2+}]_i$ was measured using a fluorescent imaging system with an epifluorescence microscope (TE 2000S; Nikon Instruments, Florence, Italy). Light of the desired wavelengths was delivered to the specimen from a xenon light source coupled to a DeltaRAM high-speed multiwavelength illuminator for excitation (Photon Technology International, Lawrenceville, NJ). The fluorescent signal was collected with an intensified CCD video camera. Regions of interest in individual cells were marked and excited at 340 and 380 nm with emission at 512nm. The 340/380 excited ratio that increased as a function of $[Ca^{2+}]_i$ was captured at 6-s intervals. Data were recorded and processed with the MetaFluor software (Universal Imaging Corp., Downingtown, PA).

Responses to CaR agonists were compared in the same cell type to those obtained after stimulation with a maximal dose of the calcium-mediated agonist ATP (100µM) that was used as internal control.

Co-immunoprecipitation

MCD4 cells were cultured in 20-cm diameter Petri dishes as described before. Cells were washed twice in ice-cold PBS and scraped into 1 ml of ice-cold EBC lysis buffer (50mM Tris pH 8.0, 120mM NaCl, 0.5% NP-40, 0.2mM sodium orthovanadate, 100mM sodium fluoride, 2mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail). Lysates were clarified by centrifugation at 13,000xg for 15 minutes at 4°C. Supernatants were pre-cleared with 100µl of protein A-sepharose suspension (Sigma) for 1 hour under rotation at 4°C. Pre-cleared lysates were incubated with 15µl of anti-Galpha12, or anti-Galpha13, or anti-RhoA antibody for 3 hours at 4°C under rotation.

Fig. 1. Expression of Calcium Sensing Receptor (CaR) in MCD4 cells (A) Reverse transcription-polymerase chain reaction (RT-PCR) amplification of CaR cDNAs expressed in MCD4 cells. Bands of 570bp corresponding to CaR were amplified from MCD4 cells as well as from mouse and rat kidney used as positive control. The oligonucleotides were designed based on the CaR nucleotide sequences straddle two different exons in the identity regions of rat, human and mouse to exclude genomic amplification. (B) Immunoblotting of MCD4 homogenate and MCD4 plasma membrane. Equally amount of blotted proteins were probed with anti-CaR affinity purified antibody. A 120kDa band corresponding to the monomeric non-glycosylated form of CaR as well as a 150kDa band corresponding to the mature glycosylated form (Gly-CaR) of the receptor were detected in MCD4 cells. Rat kidney homogenates served as positive controls.

Immunocomplexes were bound using 100µl of protein A sepharose suspension for 2 hours at 4°C and washed five times in NETN buffer (20mM Tris pH 8.0, 1mM EDTA, 900mM NaCl, 0.5% NP-40) and once in NETN buffer containing 100mM NaCl. After complete removal of the washing solution, immunocomplexes were solubilized in NuPAGE LDS sample buffer with 100mM DTT, heated at 95°C for 10 minutes and resolved on 4–12% NuPAGE gels.

After transfer to Immobilon P membrane, blotted proteins were probed with antibodies against CaR, Galpha12, Galpha13, RhoA as described above.

Statistical analysis

Data are reported as means ± SE. Statistical comparisons were made using Student's t test for the paired groups. A difference was considered significant at $p < 0.05$.

Results

CaR expression in MCD4 cells. RT-PCR and Western blotting analysis

MCD4 cells were first tested for the endogenous expression of CaR. RT-PCR performed using specific

Fig. 2. Effect of CaR agonists on $[Ca^{2+}]_i$ in renal MCD4 cells. (A) MCD4 cells loaded with Fura-2 AM 10 μ M were perfused with Ringer solutions containing CaR agonists or ATP for the time indicated by the dotted vertical bars. Fluorescence ratio 340nm/380nm was recorded and responses to CaR agonists were compared to that obtained after stimulation with a maximal dose of the calcium-mediated agonist ATP 100 μ M that was used as internal control. Each trace is representative of 3-4 different experiments. $[Ca^{2+}]_o$ 5mM induced a rapid peak of $[Ca^{2+}]_i$ corresponding to $104 \pm 8\%$ ($n=35$) with respect to the cytosolic peak induced by ATP; NPS-R 568 10 μ M induced a rapid peak of intracellular calcium that was $73 \pm 7\%$ ($n=46$, $p < 0.001$) versus ATP, while any peak was observed when stimulated cells with the enantiomer NPS-S 568 10 μ M; Gd³⁺ induced transient $[Ca^{2+}]_i$ oscillations with a maximal peak that reached $42 \pm 3\%$ ($n=43$, $p < 0.001$) of the peak induced by ATP. (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. * $P < 0.001$.

primers for the CaR yielded a 570-bp product corresponding to the expected size for CaR. A 570-bp PCR product was amplified both from rat and mouse kidneys (Fig. 1A). By immunoblotting, specific antibodies detected two major bands in MCD4 homogenate at 120kDa and 150kDa corresponding to the monomeric non-glycosylated and to the mature glycosylated form of CaR, respectively. The two bands were enriched in MCD4 plasma membrane. Rat kidney homogenates were used as positive controls (Fig. 1B).

CaR agonists increase $[Ca^{2+}]_i$ in collecting duct MCD4 cells

To evaluate whether the endogenously expressed CaR protein is a functional receptor, we examined

CaR mediated changes in $[Ca^{2+}]_i$ in MCD4 cells loaded with the fluorescent Ca²⁺ indicator Fura-2 AM (see Materials and Methods section). The cells were perfused with Ringer's solution and stimulated with three different CaR agonists: high Ca²⁺ (5mM) that has a low affinity for CaR, gadolinium (300 μ M), with an high affinity [13], and the specific allosteric modulator NPS-R 568 (10 μ M) [14]. As internal positive control, cells were stimulated with a maximal dose of the calcium-mediated agonist ATP (100 μ M) [15].

The optimal concentration of each agonist to evoke a specific response was preliminarily defined based on the respective dose response curve. The obtained EC₅₀ values were: Ca²⁺ 4.94 ± 0.49 mM; Gd³⁺ 0.22 ± 0.04 mM; NPS-R 5.28 ± 0.12 μ M. Moreover,

Fig. 3. Effect of PLC inhibition on CaR stimulation: (A) MCD4 cells loaded with Fura-2 AM 10 μ M were pretreated for 10 min with PLC inhibitor, U73122 10 μ M. Then the cells were perfused with Ringer solutions containing CaR agonists or ATP for the time indicated by the dotted vertical bars. Fluorescence ratio 340nm/380nm was recorded and responses to CaR agonists were compared in the same cell type to those obtained after stimulation with a maximal dose of the calcium mediated agonist ATP 100 μ M that was used as internal control. Each trace is representative of 3-4 different experiments. In U73122 treated cells, no increase in intracellular calcium was observed after [Ca²⁺]_o 5mM (n=143), NPS-R 10 μ M (n=38), Gd³⁺ 300 μ M (n=45) or ATP 100 μ M stimulation. (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. *P<0.001.

Fig. 4. Expression of G protein isoforms in MCD4 cells. MCD4 homogenate was blotted and probed with anti G α q, G α 12, G α 13 affinity purified antibody (1:200). All the three isoforms of the G proteins were immunodetected in MCD4 cells.

Ca²⁺ in the presence of the allosteric modulator NPS-R at 0.5 μ M exhibited a leftward shifted concentration-effect curve relative to Ca²⁺, reducing significantly the EC₅₀ value from 4.94 $\pm$ 0.49mM to 2.38 $\pm$ 0.28mM (p<0.0001; unpaired t-test; data not shown).

Fig. 2A reports a representative tracing of the fluorescence ratio 340nm/380nm in response to various agonists. Change in [Ca²⁺]_o from 1mM to 5mM caused a rapid peak of [Ca²⁺]_i which was comparable to that obtained with maximal stimulation with ATP (104 $\pm$ 8%; n=35). Stimulation with NPS-R also induced a rapid peak of intracellular calcium that was 73 $\pm$ 7% (n=46, p<0.001) with respect to ATP, whereas stimulation with its enantiomer, NPS-S 568 (10 μ M) is not able to induce [Ca²⁺]_i changes, as expected [16]. Interestingly, stimulation with Gd³⁺ induced transient [Ca²⁺]_i oscillations with

a maximal peak reaching 42 $\pm$ 3% (n= 43, p<0.001) of the peak induced by ATP. These responses were obtained in 100% of analysed cells. Together, these results confirm the presence of a functional CaR in MCD4 cells.

Increase in intracellular calcium induced by CaR activation is mediated by a PLC-dependent pathway

To evaluate whether the increase in intracellular calcium induced by CaR activation involves a PLC-dependent pathway, cells were stimulated with CaR agonists in the presence of the PLC inhibitor U73122. Cells were first checked for their responsiveness to the CaR agonists (not shown). Cells were then treated with the PLC inhibitor U73122 10 μ M for 10 min, loaded with Fura-2 AM, perfused with Ringer's solution and stimulated

Fig. 5. Effect of Rho inhibition on CaR activated calcium signaling. (A) MCD4 cells were pretreated for 4 h with the permeable Rho inhibitor C3 transferase (10µg/ml). Cells were then loaded with Fura-2 AM and perfused with Ringer solutions containing CaR agonists or ATP for the time indicated by the dotted vertical bars. Fluorescence ratio 340nm/380nm was recorded and responses to CaR agonists were compared in the same cell type to those obtained after stimulation with a maximal dose of the calcium mediated agonist ATP 100µM that was used as internal control. Each trace is representative of 3-4 different experiments. $[Ca^{2+}]_o$ 5mM induced a rapid peak of $[Ca^{2+}]_i$ corresponding to $125 \pm 16\%$ ($n=77$, $p<0.001$) with respect to the response obtained with ATP. In contrast, the response to Gd^{3+} was completely abolished in cells pretreated with Rho inhibitor C3 transferase ($n=98$). (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. * $P<0.001$.

with the following agonists $[Ca^{2+}]_o$ 5mM ($n=143$), NPS-R 568 10µM ($n=38$), Gd^{3+} 300µM ($n=45$), respectively.

U-73122 completely blocked $[Ca^{2+}]_i$ changes after stimulation with each CaR agonist. As expected, the response to ATP was abolished as well (Fig. 3A).

These data demonstrate that, in MCD4 cells, CaR is functionally expressed and activates a signal transduction pathway that is calcium-mediated and PLC-dependent.

CaR-mediated transient $[Ca^{2+}]_i$ oscillations are Rho-dependent

CaR is a pleiotropic receptor and its activation is associated with different intracellular calcium signaling depending on the specific type of G protein to which it is coupled [3, 14, 17-21]. Besides Gq, it has been demonstrated that CaR can be coupled to G12/13 protein [20]. To this respect, it has been shown that CaR agonists, such as amino acids, induce intracellular calcium oscillations mediated by activation of Gα12 and the monomeric GTPase Rho. [22, 23].

Therefore, the next step was to test the hypothesis that calcium oscillations induced by Gd^{3+} in MCD4 cells are the consequence of activation of G12/13 protein-Rho pathway. First, we analyzed the expression of G12/13 proteins in MCD4. Besides the presence of Gαq, both

Gα12 and Gα13 proteins were immunodetected in MCD4 cell homogenate (Fig. 4).

Next, changes in $[Ca^{2+}]_i$ were evaluated in cells pretreated with the permeable Rho inhibitor C3 transferase (10µg/ml) for 4 h. CaR was stimulated with high extracellular calcium (5mM) or with Gd^{3+} (300µM). Interestingly, the response to Gd^{3+} was abolished in cells pretreated with Rho inhibitor C3 transferase ($n=98$), whereas the response to 5mM $[Ca^{2+}]_o$ was not affected ($125 \pm 16\%$ compared with ATP, $n=77$, $p<0.001$, Fig. 5A).

These results demonstrate that calcium oscillations are associated with Gd^{3+} activation and involve a G12/13 protein-Rho dependent pathway.

A downstream effector of Rho is the Rho-associated coiled coil forming protein serine/threonine kinase [24]. To explore whether ROK is involved in Gd^{3+} induced calcium oscillation as a downstream effector of Rho, CaR mediated changes in $[Ca^{2+}]_i$ were examined in cells pretreated with Y27632 (10µM), a highly potent, cell-permeable, selective ROK inhibitor. Y27632 selectively abolished the response to Gd^{3+} ($n=64$), whereas increasing the concentration of $[Ca^{2+}]_o$ (from 1mM to 5mM) induced a cytosolic peak of calcium, as in non treated cells ($113 \pm 16\%$ compared with ATP, $n=19$, Fig. 6A).

These results demonstrate that in MCD4 cells, stimulation of CaR with Gd^{3+} activates a distinct signal

Fig. 6. Effect of Rho kinase inhibition on CaR calcium signaling: (A) MCD4 cells loaded with Fura-2 AM were pretreated with the selective ROK kinase (Rho-associated coiled coil forming protein serine/threonine kinase) inhibitor, Y27632 (10 μ M). Cells were then perfused with Ringer solutions containing CaR agonists or ATP for the time indicated by the dotted vertical bars. Fluorescence ratio 340nm/380nm was recorded and responses to CaR agonists were compared in the same cell type to those obtained after stimulation with a maximal dose of the calcium mediated agonist ATP 100 μ M that was used as internal control. Each trace is representative of 3-4 different experiments. 5mM [Ca²⁺]_o induced a rapid peak of [Ca²⁺]_i that respect to cytosolic peak induced by ATP were 113 $\pm$ 16% (n=19, p<0.001), while Gd³⁺ (n= 64) was unable to induce any increase in intracellular calcium. (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. *P<0.001.

Fig. 7. Effect of store depletion on CaR stimulation with Gd³⁺. (A) MCD4 cells loaded with Fura-2 AM were perfused with Ringer solutions containing 5 μ M cyclopiazonic acid (CPA), inhibitor of the sarcoplasmic reticulum Ca²⁺-pump and CaR agonists or ATP for the time indicated by the dotted vertical bars. Fluorescence ratio 340nm/380nm was recorded and responses to CaR agonists were compared in the same cell type to those obtained after stimulation with a maximal dose of the calcium mediated agonist ATP 100 μ M that was used as internal control. The trace is representative of 3 different experiments. Gd³⁺ (n=67) and ATP did not induce any increase in intracellular calcium in CPA pretreated cells. (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. *P<0.001.

transduction pathway that is calcium mediated and Rho-ROK dependent.

CaR mediated transient [Ca²⁺]_i oscillations require calcium mobilization from internal stores

It has been shown that CaR responds to extracellular calcium and aromatic amino acids with the production of different patterns of oscillations in intracellular calcium concentrations. In particular, Rey et al. showed that calcium oscillations stimulated by aromatic amino acids were selectively abolished by TRPC1 down-regulation

Fig. 8. Effect of calcium channels inhibition on CaR stimulation with Gd^{3+} . (A) MCD4 cells loaded with Fura-2 AM were perfused with Ringer's solution containing 10 μ M ruthenium red, generic inhibitor of calcium channels. Then, cells were exposed to 5mM Ca^{2+} ($115\pm 19\%$, $n=108$; n.s. compared to ATP) and Gd^{3+} ($69\pm 5\%$, $n=45$, $p<0.001$ compared to ATP). (B) Means $\pm$ SE. Changes are reported as percentage of the maximal response to ATP. * $P<0.001$.

Fig. 9. Coimmunoprecipitation studies and Western blotting: MCD4 cells were solubilized and lysates were subjected to immunoprecipitation (IP) using $G\alpha 12$, $G\alpha 13$ or RhoA antibodies. Immuno-complexes were immunoblotted and revealed with CaR, $G\alpha 12$, $G\alpha 13$ or RhoA antibodies. As a control, cell lysates were loaded and probed with the respective antibodies. This representative experiment was performed three times with similar results.

[25]. To investigate the mechanism that regulates intracellular calcium oscillations upon stimulation with Gd^{3+} , we first analyzed whether Gd^{3+} -induced calcium oscillations require mobilization from internal stores.

To this end, cells were perfused with a solution containing 5 μ M cyclopiazonic acid (CPA), an inhibitor of the sarcoplasmic reticulum Ca^{2+} -pump. This causes calcium store depletion. Under these experimental

conditions, both Gd^{3+} - and ATP-dependent calcium oscillations were prevented (Fig. 7A, $n=67$). This result indicates that Gd^{3+} stimulates calcium release from intracellular stores.

Accordingly, generic inhibition of membrane calcium channels 10 μ M Ruthenium red did not prevent calcium changes both in response to 5mM $[Ca^{2+}]$ ($115 \pm 19\%$, $n=108$, $p<0.001$) and Gd^{3+} ($69 \pm 5\%$, $n=45$, $p<0.001$) stimulation, indicating that intracellular calcium increases are not due to calcium entry from the extracellular environment (Fig. 8A).

To evaluate whether CaR is functionally coupled to the $G\alpha_{12/13}$ -Rho pathway, the possible protein-protein interaction between these proteins was investigated by co-immunoprecipitation studies. Interestingly, both CaR and RhoA co-immunoprecipitated with $G\alpha_{12}$ and with $G\alpha_{13}$ (Fig. 9). Conversely, both CaR and $G\alpha_{13}$ co-immunoprecipitated with RhoA, whereas only CaR and not $G\alpha_{12}$ co-immunoprecipitated with RhoA. These data suggest that $G\alpha_{13}$ is likely the GTPase to which CaR preferentially couples to activate RhoA resulting in calcium oscillations.

Discussion

In this report, we provide evidence demonstrating the endogenous expression of functional CaR in a mouse renal cortical collecting duct cell line [12]. CaR expression was first checked at mRNA and protein level by RT-PCR and Western blotting. Next, release of intracellular calcium in response to extracellular Ca^{2+} or Gd^{3+} provided pharmacological evidence that the immunoreactive protein was indeed a functional receptor.

CaR is highly expressed in the kidney, both on the apical and basolateral membranes of epithelial tubular cells, leading it to respond to signals deriving both from the tubular fluid and the interstitial plasma [21]. It has been suggested that CaR might couple to different G proteins according to the cell type. Upon binding, Ca^{2+} activates the G-proteins, $G_{q/11}$, G_i , and $G_{12/13}$, which in turn stimulate phospholipase C (PLC), resulting in the production of diacylglycerol and inositol triphosphate. As a consequence, calcium is released from intracellular stores causing adenylate cyclase inhibition and Rho kinase activation, respectively [9]. Besides adenylate cyclase inhibition, CaR can also reduce cAMP levels by phosphodiesterase activation [10]. In some cell types, however, CaR has been shown to activate G_s , thus resulting in adenylate cyclase stimulation [11].

In this scenario, the lack of cellular models endogenously expressing functional CaR makes it difficult to identify the signaling associated with CaR in a specific renal cell type.

In this study, we identified and dissected the signal transduction pathway(s) activated by CaR in mouse renal cortical collecting duct cells [12] expressing functional CaR. The physiological relevance of the data obtained lies in the endogenous expression of a functional receptor in these cells.

The specific interest in analyzing CaR signaling in collecting duct principal cells stems from the peculiar apical expression of CaR in this nephron segment. Indeed, the CaR protein is basolateral in the thick ascending limb of Henle's loop and luminal in the proximal tubule and collecting duct, suggesting that the receptor senses calcium changes occurring both within the interstitial plasma and the urinary space [26, 6].

This likely facilitates a local regulation of physiological processes without the need to evoke systemic changes in plasma composition.

In renal collecting duct MCD4 cells, we analyzed the response to several CaR agonists including the physiological agonist Ca^{2+} 5mM, the allosteric modulator NPS-R 568 and the trivalent cation Gd^{3+} .

We found that CaR-induced increases in intracellular calcium were prevented by pre-treatment with the PLC inhibitor U73122, indicating that in MCD4 cells, an intracellular rise in calcium is PLC-dependent, likely due to coupling of CaR with the Gq protein.

A new result was the observation that receptor activation produced a distinct profile in terms of the increase in intracellular calcium in response to the three agonists: Ca^{2+} 5mM and NPS-R 568 induced a rapid transient calcium peak, whereas Gd^{3+} stimulation resulted in calcium oscillations. These responses can be considered specifically CaR mediated for at least two reasons: first, stimulation with NPS-S 568, the inactive enantiomeric form of the allosteric modulator NPS-R 568, does not modify $[Ca^{2+}]_i$ levels. Second, EC50 value of Ca^{2+} in the presence of NPS-R 0.5 μ M relative to EC50 value of Ca^{2+} shows a significant decrease of around 50%.

The different profiles in terms of the increase in intracellular calcium may be due to coupling to distinct G proteins. Indeed, it has been shown that CaR induces a different calcium signaling dependent on the G-protein to which it is coupled. Specifically, calcium oscillations have been shown to involve activation of the monomeric G protein Rho. Transient intracellular calcium oscillations elicited by CaR in response to amino acids involve

Fig. 10. Proposed model of two distinct pathways of CaR activation: in renal collecting duct MCD4 cells extracellular agonists activate different G-protein-coupled CaR, leading to increases in $[Ca^{2+}]_i$. (a) High extracellular calcium and NPS induce $[Ca^{2+}]_i$ peak via Gq-protein coupled CaR. (b) Gd^{3+} activates Rho/ROK-pathways via $G\alpha_{12/13}$ -protein, causes characteristic $[Ca^{2+}]_i$ oscillations. Both these changes in $[Ca^{2+}]_i$ are mainly due to Ca^{2+} release from intracellular stores.

activation of the heterotrimeric G12/13 protein [20], the small GTPase Rho, the scaffolding protein filamin A and the cytoplasmic tail of CaR [22, 23]. In turn, Rho can activate PLC epsilon [27] with consequent calcium release from internal stores [28-31].

The results presented here demonstrate that in our model, the inhibition of Rho with the permeable C3 transferase or with a ROK inhibitor abolished intracellular calcium oscillations induced by Gd^{3+} , while the peak induced by 5mM $[Ca^{2+}]_o$ was unaffected. On the other hand, emptying the intracellular calcium stores selectively abolished the response to Gd^{3+} .

This indicates that in collecting duct MCD4 cells, CaR stimulation with distinct agonists activates two distinct transduction pathways, both of which are PLC-dependent. The transient $[Ca^{2+}]_i$ oscillations produced by Gd^{3+} are due to calcium mobilization from internal stores and are modulated by Rho-ROK signaling, whereas the rapid peak of $[Ca^{2+}]_i$ in response to 5mM $[Ca^{2+}]_o$ is mainly due to Ca^{2+} released from intracellular stores subsequent CaR/Gq activation.

To explain the effect of ROK inhibitor in preventing transient $[Ca^{2+}]_i$ oscillations, one possibility is that under physiological conditions ROK is required for Ins (1,4,5) P_3 (IP3) receptor phosphorylation, essential for calcium release from intracellular stores. Indeed, several lines of evidence support the hypothesis that $[Ca^{2+}]_i$ oscillations generated in response to GPCR activation via the intracellular second messenger IP3 depend on the regulation of the IP3 receptor [32-34].

In particular, it has been reported that RhoA-mediated ROK activity increases phosphorylation of three different IP3 receptors, known collectively as IP3-gated

calcium channels. This subsequently stimulates IP3-mediated calcium flux [24]. This has also been demonstrated for another GPCR receptor (CD44, a hyaluronan (HA) receptor) that induces release of calcium from internal stores after activation of G12/13-Rho-PLCepsilon-IP3. So we can suppose that the involvement of ROK in oscillation induced by Gd^{3+} probably depends on the modulation of the IP3 receptor.

It has been shown that calcium oscillations can also be associated with activation of TRPC calcium channels [25]. This, however, seems to be unlikely in MCD4 cells, based on our findings demonstrating that CPA, an inhibitor of the sarcoplasmic reticulum Ca^{2+} -pump, prevents Gd^{3+} induced calcium oscillations whereas ruthenium red, a generic inhibitor of calcium channels [35] does not affect calcium oscillations in response to Gd^{3+} . These data favor the hypothesis that Gd^{3+} induces calcium oscillations subsequent to release from intracellular stores.

In summary, we provide evidence that in collecting duct MCD4 cells, CaR activation with Ca^{2+} and Gd^{3+} acting on distinct sites of the receptor [36] leads to the activation of two different G proteins: Gq and G12/13, respectively. This results in activation of dual signaling pathways, both of which are PLC-dependent. In fact, the transient $[Ca^{2+}]_i$ oscillations produced by Gd^{3+} and $[Ca^{2+}]_i$ peaks by 5mM calcium are due to calcium mobilization from internal stores and are modulated by Rho-Rho kinase signaling and PLC-IP3 pathway, respectively (see proposed model in Fig. 10).

The precise mechanism and components mediating the generation of two different calcium signals require further investigation.

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