Original Paper

Calcium-Sensing Receptor Stimulation in Cultured Glomerular Podocytes Induces TRPC6-Dependent Calcium Entry and RhoA Activation

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Key Words

Trpc6 • Calcium-sensing receptor • RhoA • Podocytes

Abstract

Background/Aims: Recent studies provided compelling evidence that stimulation of the calcium sensing receptor (CaSR) exerts direct renoprotective action at the glomerular podocyte level. This protective action may be attributed to the RhoA-dependent stabilization of the actin cytoskeleton. However, the underlying mechanisms remain unclear. **Methods:** In the present study, an immortalized human podocyte cell line was used. Fluo-3 fluorescence was utilized to determine intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), and western blotting was used to measure canonical transient receptor potential 6 (TRPC6) protein expression and RhoA activity. Stress fibers were detected by FITC-phalloidin. **Results:** Activating CaSR with a high extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) or R-568 (a type II CaSR agonist) induces an increase in the $[\text{Ca}^{2+}]_i$ in a dose-dependent manner. This increase in $[\text{Ca}^{2+}]_i$ is phospholipase C (PLC)-dependent and is smaller in the absence of extracellular Ca^{2+} than in the presence of 0.5 mM $[\text{Ca}^{2+}]_o$. The CaSR activation-induced $[\text{Ca}^{2+}]_i$ increase is attenuated by the pharmacological blockage of TRPC6 channels or siRNA targeting TRPC6. These data suggest that TRPC6 is involved in CaSR activation-induced Ca^{2+} influx. Consistent with a previous study, CaSR stimulation results in an increase in RhoA activity. However, the knockdown of TRPC6 significantly abolished the RhoA activity increase induced by CaSR stimulation, suggesting that TRPC6-dependent Ca^{2+} entry is required for RhoA activation. The activated RhoA is involved in the formation of stress fibers and focal adhesions in response to CaSR stimulation because siRNA targeting RhoA attenuated the increase in the stress fiber mediated by CaSR stimulation. Moreover, this effect of CaSR activation on the formation of stress fibers is also abolished by the knockdown of TRPC6. **Conclusion:** TRPC6 is involved in the regulation of stress fiber formation and focal adhesions via the RhoA pathway in response to CaSR activation. This may explain the direct protective action of CaSR agonists.

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Introduction

The calcium-sensing receptor (CaSR) was originally cloned from parathyroid glands, where it tightly regulates parathyroid hormone secretion in response to changes in extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) [1, 2]. Pharmacological activation of CaSR can be achieved by phenylalkylamine compounds, such as R-568 and cinacalcet. Cinacalcet is currently prescribed for the treatment of secondary hyperparathyroidism in patients with end-stage renal disease. Surprisingly, animal experiments showed that these agents have renoprotective effects beyond the control of calcium homeostasis [3]. In the kidney, CaSR is well-known for regulating calcium secretion and absorption in renal tubules [4]. However, accumulating evidence indicates that CaSR is expressed in glomeruli as well, including mesangial cells and podocytes [5]. Observations from our group and others show that CaSR is expressed in mesangial cells and regulates cell proliferation [6, 7]. Recently, two studies provided compelling evidence that pharmacological activation of CaSR by the calcimimetic R-568 exerts a direct renoprotective effect at the glomerular podocyte level [8, 9]. This finding may provide a novel treatment option for delaying progressive renal failure. However, very little is currently known about the signaling cascades initiated by CaSR activation in podocytes.

CaSR is a member of the group C G protein-coupled receptor (GPCR) superfamily. Elevations in $[\text{Ca}^{2+}]_o$ or exposure to other CaSR agonists commonly elicit an intracellular calcium signal through interactions between the CaSR and phospholipase C (PLC) [1, 10]. These interactions result in the hydrolysis of phosphatidylinositol-4, 5-bisphosphate by PLC to form inositol-1, 4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which lead to an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) through the release of IP_3 -sensitive calcium stores, through the Ca^{2+} influx or both [1, 10]. Relatively little is known about the Ca^{2+} influx mechanism upon CaSR activation. Canonical transient receptor potential (TRPC) channels, nonselective cation channels, seem to be responsible for Ca^{2+} entry induced by CaSR activation in some cell types, such as salivary ductal cells [11], vascular smooth muscle cells [12, 13], keratinocytes [14], MCF-7 breast cancer cells [15] and glomerular mesangial cells [6].

TRPC1, TRPC3, TRPC5 and TRPC6 are expressed in podocytes [16, 17]. TRPC6, a predominant member of these TRPC channels, plays a vital role in the regulation of cell functions, especially in dynamic actin cytoskeleton regulation [18, 19, 20]. Upon stimulation with angiotensin II or high glucose in podocytes, a TRPC6-mediated Ca^{2+} influx increases the activity of RhoA, a small Rho GTPase, thereby leading to stress fiber formation to induce a stationary homeostatic podocyte phenotype [16, 21]. Interestingly, the renoprotective effects of CaSR agonists from cytoskeleton damage is associated with RhoA activity because treatment with the Rho-kinase inhibitor H1152, but not ERK1/2, p38 and JNK inhibitors, abolished R-568-induced increases in stress fiber and focal adhesion formation [9]. Therefore, in the present study, we address whether a TRPC6-dependent Ca^{2+} influx can be induced by the activation of CaSR and if so, whether it can mediate RhoA activation and be involved in actin cytoskeleton changes induced by CaSR activation.

Materials and Methods

Cell Culture and Transfection

An immortalized human podocyte cell line was provided by Dr. Moin Saleem of the Children's Renal Unit and Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK. The cells were cultured as described previously [22]. Briefly, cells were grown at 33°C in an RPMI-1640 medium (HyClone, Logan, UT, USA; SH30809.01B) supplemented with ITS (Sigma-Aldrich Co., St. Louis, MO, USA; 13146-5 ML) and 10% fetal bovine serum (FBS; ScienCell, San Diego, CA, USA; 0500). Human podocytes between passages 4 and 15 were used. To induce differentiation, the cells were cultured at 37°C in the same medium in a 5% CO_2 humidified atmosphere for 10-14 days, and the culture medium was changed every 4 days. The human breast

cancer cell line MCF-7 was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and maintained in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM, HyClone; SH30022.01B) containing 10% FBS. The cells were transiently transfected with TRPC6 siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-42672, 10 µM), RhoA siRNA (Santa Cruz Biotechnology, Inc.; sc-29471, 10 µM) or scrambled siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-37007, 10 µM) using the X-treme GENE siRNA Transfection Reagent (Roche, Basel, Switzerland; 04476093001) according to the manufacturer's instructions. Assays on the transfected cells were conducted within 24–48 h post-transfection.

Reverse-transcription PCR

Reverse transcription was performed using an RT system (Eppendorf Mastercycler, Hamburg, Germany) in a 10 µl reaction mixture. A High Capacity cDNA RT Kit (ABI Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesis according to the manufacturer's instructions. The primer sequences used to amplify CaSR were as follows (5'-3'): sense CGGGGTACCTTAAGCACCTACGGCATCTAA and antisense GCTCTAGAGTTAACGCGATCCCAAAGGGCTC. All primers were synthesized by Shanghai Invitrogen Corporation. The PCR cycling conditions for CaSR included one cycle of 10 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 60°C, and 60 s at 72°C; and one cycle of 10 min at 72°C. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Reverse transcriptase was omitted as a negative control for the reaction to eliminate amplification from contaminating genomic DNA.

Western blotting

Western blotting was performed using a standard protocol. Human podocytes were starved for 24 h in serum-free medium before being stimulated with R-568 (a selective II type CaSR agonist, Tocris Bioscience, Minneapolis, MN, USA; 177172-49-5, DMSO to 100 mM). In calcium-free medium, the calcium ion chelator ethylenedinitrilotetraacetic acid (EGTA, Sigma-Aldrich Co.; E0396-10 g) was added to obtain a final concentration of 1.0 mM. Anti-CaSR (1:200, Santa Cruz Biotechnology, Inc.; sc-33821 200 µg, IgG in 1.0 mL of PBS), anti-TRPC6 (1:200, Alomone Labs, Israel; ACC-017 200 µL), anti-RhoA (1:500, Santa Cruz Biotechnology, Inc.; sc-418), and anti-actin antibodies (1:1000, ZSGB-Bio, Beijing, China; ZS-TA-09) were used as primary antibodies. Fluorescence-conjugated goat anti-rabbit (1:10000, LI-COR Biosciences, Lincoln, NE, USA; 926-32211) or goat anti-mouse (1:10, 000, LI-COR Biosciences; 926-32210) IgG antibodies were used as secondary antibodies. Band intensities were quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

Immunofluorescence

Immunofluorescence staining was conducted on cultured podocytes grown on coverslips using a standard protocol. Briefly, after washing, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.4% Triton X-100 in PBS for 60 min at room temperature. Nonspecific binding sites were blocked with 50% goat serum in PBS for 30 min at 37°C. After washing with blocking buffer (PBS), the cells were incubated with the appropriate antibody dilutions. For CaSR staining, the cells were incubated with anti-CaSR antibody (1:50) overnight at 4°C. After washing, the cells were stained with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1:500; Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. The nuclei were counterstained with DAPI (1:50; BiYunTian, Beijing, China; C1005-10 ml) for 15 min at room temperature. After washing, the samples were examined under a laser scanning confocal microscope (Olympus, Tokyo, Japan). Calibrations were performed immediately following each experiment. More than 40 cells were inspected per experiment, and photographs of cells with typical morphology and staining are presented. At least three independent experiments were conducted.

For cytoskeleton staining, human podocytes were starved for 24 h in a serum-free medium prior to the experiment. Cells were treated for 30 min with R-568 (10 nmol/mL). In addition, cells were transfected with TRPC6 siRNA or RhoA siRNA in combination with R-568. The cells were subsequently fixated and stained with vinculin antibodies (1:500, Sigma-Aldrich Co.; v9131-100µL) and the secondary antibody (1:600, Thermo Fisher Scientific Inc. Waltham, MA, USA; Alexa Fluor 594; Z25007). After that, the cells were stained with FITC-phalloidin (1:200 Sigma-Aldrich Co.; p5282-1mg) and then washed 5 times with PBS. The cytoskeleton fluorescence was visualized using a fluorescence microscope (Olympus, Tokyo, Japan). More than 50 cells were inspected per experiment, and three independent experiments were conducted. Quantification analysis of the number of stress fibers was performed, as described previously [16]. Stress

fibers were counted manually in each individual cell in independent images. A stress fiber was defined as a phalloidin-positive structure that transverses the entire cell.

Fluorescence measurement of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

Human podocytes were grown on coverslips and loaded in 1% physiological saline solution containing Pluronic F-127 (0.03%, Sigma-Aldrich Co.; p2443-1kg) and Fluo-3/AM (3 μM , Invitrogen Co., Carlsbad, CA, USA; F1242) at 37°C for 45 min. After washing, the coverslips with cells were placed in a chamber containing HEPES-buffered Na^+ medium (HBM) containing the following (in mM): 137 NaCl, 5 KCl, 0.5 CaCl_2 , 1.2 MgCl_2 , 0.44 KH_2PO_4 , 4.2 NaHCO_3 , 10 glucose, and 20 HEPES; the pH was adjusted to 7.4 with NaOH. For Ca^{2+} -free HBM, Ca^{2+} was omitted. Then, the podocytes were stimulated with a variety of agonists, antagonists or inhibitors as described in the results, including R-568 (a selective II type CaSR agonist, Tocris Bioscience, Minneapolis, MN, USA; 177172-49-5, DMSO to 100 mM), NPS2390 (an antagonist of CaSR, Sigma-Aldrich Co.; N4786), U73122 (a PLC inhibitor, Sigma-Aldrich Co.; U6756-5MG), U73343 (an inactive analog of U73122, Sigma-Aldrich Co.; U6881-5MG), 2-aminoethoxydiphenyl borate (2-APB, a nonselective TRPC6 blocker, Sigma-Aldrich Co.; D9754-1G), and SKF-96365 (a nonselective TRPC6 blocker, Sigma-Aldrich Co.; S7809-5MG). The fluorescence intensity of Fluo-3 in the cells was recorded with a laser confocal scanning microscope (FV300; Olympus, Japan) $[\text{Ca}^{2+}]_i$ was expressed as a pseudo-ratio value of the actual fluorescence intensity divided by the average baseline fluorescence intensity. Calibrations were performed immediately following each experiment. Data from 20 to 30 cells were summarized in a single run, and at least three independent experiments were conducted.

RhoA activation assay

RhoA activity was measured using a Rho activation assay kit (Cytoskeleton, Inc., Denver, CO, USA; BK036-S) according to the manufacturer's instructions. Human podocytes were starved for 24 h in serum-free medium before being stimulated with R-568. The cells were lysed in cell lysis buffer, and the lysates were immediately clarified by centrifugation at 10,000 $\times g$, 4°C for 1 min. The supernatants were incubated with Rhotekin-Rho binding domain (RBD) glutathione affinity beads, which specifically bind to GTP-bound RhoA. The beads were washed, and the immunoprecipitated complex was resuspended in a 2 $\times$ Laemmli sample buffer and subjected to 12% SDS-PAGE, followed by western blot analysis. The total RhoA protein was determined and used to normalize GTP-bound RhoA densitometric units. At least three independent experiments were conducted.

Statistical analysis

Data are presented as the mean $\pm$ standard error (SEM). The significance of differences between groups was assessed using two-tailed Student's *t*-test or one-way ANOVA followed by Dunnett's test. A *p*-value < 0.05 was considered to be statistically significant.

Results

CaSR is present in the cultured human podocytes

First, we examined whether CaSR is expressed in our cultured human podocytes. As shown in Fig. 1A, a 424-bp PCR product was detected after RT-PCR using CaSR-specific primers. In the group without reverse transcriptase (RT⁻), this amplification product was not detected, indicating that there was no genomic DNA contamination in the RNA samples. A PCR product with the same size as the product from human podocytes was detected in the human breast cancer cell line MCF-7, which was used as a positive control. As shown in Fig. 1B, western blot analysis using a CaSR-specific antibody detected a 160-kDa protein band, representing the mature glycosylated form of CaSR, in both human podocytes and the control MCF-7 cell line. As shown in Fig. 1C, immunofluorescence revealed that CaSR was primarily localized to the cell membrane and cytosol of podocytes. These results determined that the cultured human podocytes express CaSR mRNA and protein.

Fig. 1. *In vitro* cultured human podocytes express CaSR mRNA and protein. (A) RT-PCR was performed on total RNA extracted from podocytes and MCF-7 cells, a human breast cancer cell line, were used as a positive control. Specific primers were used to assess CaSR mRNA expression. A PCR product with an expected size of 424 bp was detected. In the sample without reverse transcriptase (RT-), no PCR products were detected, PODO: podocytes. (B) Western blotting was used to detect CaSR protein expression in human podocytes. A 160-kD band was detected in the podocytes and MCF-7 cells. (C) Immunofluorescence using an anti-CaSR antibody detected CaSR expression in podocytes. Red fluorescence indicates labeled CaSR, and blue fluorescence indicates nuclei. Scale bar: 20 μ m. At least 40 cells were observed. A representative image is shown.

Activation of CaSR stimulates an increase $[Ca^{2+}]_i$ in human podocytes

To evaluate whether the expression of CaSR protein is associated with the presence of functional receptors, Fluo-3/AM-loaded human podocytes were stimulated by Ca^{2+} (a type I CaSR agonist) and the calcimimetic compound R-568 (a type II CaSR agonist). As shown in Fig. 2A, the $[Ca^{2+}]_o$ change from 1 mmol/L to 3 mmol/L triggered a rapid increase in $[Ca^{2+}]_i$ to a peak level, which was maintained for more than 300 seconds. The elevated $[Ca^{2+}]_o$ elicited increases in $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 2B). Stimulation of podocytes by 10 nmol/L R-568 produced a response similar to that of 3 mmol/L $[Ca^{2+}]_o$ stimulation (Fig. 2C). In addition, the $[Ca^{2+}]_i$ increase induced by 10 nmol/L R-568 was dose dependent (Fig. 2D). Furthermore, the $[Ca^{2+}]_i$ increase induced by either 3 mmol/L $[Ca^{2+}]_o$ or 10 nmol/L R-568 was inhibited by pre-incubation with the 10 μ mol/L CaSR antagonist NPS2390 for 30 min (Fig. 2E and 2F), demonstrating that CaSR activation is not agonist specific. To evaluate whether the increase in $[Ca^{2+}]_i$ induced by CaSR activation involves a PLC-dependent pathway, cells were stimulated with CaSR agonists in the presence of the PLC inhibitor U73122 or U73343, an inactive analog of U73122. The $[Ca^{2+}]_i$ increase induced by 3 mM $[Ca^{2+}]_o$ or 10 nmol/L R-568 was significantly inhibited by pretreatment with 30 μ M U73122, as shown in Fig. 2G and 2H, respectively. However, 30 μ M U73343 did not inhibit the increase of $[Ca^{2+}]_i$ induced by 3 mM $[Ca^{2+}]_o$ or 10 nmol/L R-568, as shown in Fig. 2G and 2H. Taken together, these data confirmed that CaSR protein is functionally expressed in human podocytes and activates a PLC-dependent $[Ca^{2+}]_i$ increase.

Activation of CaSR induces TRPC6-dependent Ca^{2+} influx

Next, we investigated whether CaSR activation induces Ca^{2+} influx in human podocytes. Podocytes were stimulated with R-568 in the absence of extracellular Ca^{2+} . As shown in Fig. 3A, 10 nmol/L R-568 induced a transient $[Ca^{2+}]_i$ increase in podocytes. However, the R-568-induced $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+} was smaller than that observed in the presence of 0.5 mM $[Ca^{2+}]_o$ and had no subsequent sustained increase in $[Ca^{2+}]_i$ (Fig. 3B), suggesting that an extracellular Ca^{2+} influx is most likely involved in the increase of $[Ca^{2+}]_i$ by CaSR agonists. To evaluate whether TRPC6 contributes to $[Ca^{2+}]_i$ increase induced by CaSR activation, we transfected human podocytes with specific siRNA to downregulate

Fig. 2. CaSR activation by Ca^{2+} and R-568 results in a $[Ca^{2+}]_i$ increase. Confocal microscopy using Fura-3 fluorescent dye was used to observe changes in the Ca^{2+} concentration. (A) Change in $[Ca^{2+}]_o$ from 0.5 mmol/L to 3 mmol/L induced a rapid increase to a peak level, which was maintained for more than 300 seconds after the peak. (B) Extracellular Ca^{2+} (0.5–20 mmol/L) induced a $[Ca^{2+}]_i$ increase in podocytes in a dose-dependent manner; data are shown as the mean $\pm$ SE of 20–30 cells. (C) In the presence of extracellular Ca^{2+} at 0.5 mmol/L, the addition of 10 nmol/L R-568 induced a rapid increase in $[Ca^{2+}]_i$ to a peak level and a slower decrease. (D) In the presence of extracellular Ca^{2+} at 0.5 mmol/L, the addition of various doses of R-568 (3, 5, 10 nmol/L) induced $[Ca^{2+}]_i$ increases in a dose-dependent manner. (E, F) 10 μ mol/L of the CaSR antagonist NPS2390 significantly inhibited $[Ca^{2+}]_i$ increase induced by 3 mmol/L $[Ca^{2+}]_o$ or 10 nmol/L R-568 ($p < 0.01$ vs. NPS2390 (-), $n = 3$). (G, H) 30 μ mol/L of the PLC inhibitor U73122 inhibited $[Ca^{2+}]_i$ increase induced by 3 mmol/L $[Ca^{2+}]_o$ or 10 nmol/L R-568 compared with U73343, an inactive analog of U73122. ($p < 0.01$ vs. U73343, $n = 3$) Number of trials = 3; 20–30 cells were selected in each trial.

the expression of TRPC6. The efficiency of TRPC6-siRNA was confirmed by western blot analyses (Fig. 3C). Compared with cells transfected with scrambled siRNA, transfection with TRPC6 siRNA partially, but significantly, inhibited the R-568-induced $[Ca^{2+}]_i$ increase by 37.94% ($p < 0.001$, $n = 3$; Fig. 3D). TRPC6 knockdown also attenuated the $[Ca^{2+}]_o$ -induced

Fig. 3. TRPC6 participates in CaSR activation-induced $[\text{Ca}^{2+}]_i$ increase. Confocal microscopy using Fura-3 fluorescent dye was used to observe changes in the Ca^{2+} concentration. (A) In extracellular fluid lacking calcium, the addition of R-568 to a final concentration of 10 nmol/L induced a transient increase in $[\text{Ca}^{2+}]_i$. (B) In the presence of extracellular Ca^{2+} at 0.5 mmol/L, the addition of 10 nmol/L R-568 induced a rapid increase in $[\text{Ca}^{2+}]_i$ to a peak level and a slower decrease. (C) Western blot experiments showed that transfection with TRPC6 siRNA reduced TRPC6 protein expression (** $p < 0.001$ vs. Scr, $n = 3$). (D) TRPC6 siRNA transfection inhibited calcium signal induced by 10 nmol/L R-568 (** $p < 0.001$ vs. Scr, $n = 3$). (E) TRPC6 siRNA transfection inhibited calcium signal induced by 3 mmol/L $[\text{Ca}^{2+}]_o$ (** $p < 0.01$ vs. Scr). (F) The 3 mM $[\text{Ca}^{2+}]_o$ -induced $[\text{Ca}^{2+}]_i$ increase is inhibited in the presence of TRPC channel blockers (** $p < 0.001$ vs. Ctrl, $n = 3$). Cells were pretreated with 50 μM SKF-96365 or 100 μM 2-APB for 20 min in a medium containing 0.5 mM Ca^{2+} followed by a change to 3 mM. (G) The 10 nmol/L R-568-induced $[\text{Ca}^{2+}]_i$ increase is inhibited in the presence of TRPC channel blockers (** $p < 0.001$ vs. Ctrl, $n = 3$). Cells were pretreated with 50 μM SKF-96365 or 100 μM 2-APB for 20 min in medium containing 0.5 mM Ca^{2+} followed by the addition of 10 nmol/L R-568. Number of trials = 3; 20–30 cells were selected in each trial. +SKF96365: podocytes pretreated with SKF96365 for 20 min. +2-APB: podocytes pretreated with 2-APB for 20 min.

$[\text{Ca}^{2+}]_i$ increase (Fig. 3E). Transfection with scrambled siRNA did not alter $[\text{Ca}^{2+}]_o$ - or the R-568-induced $[\text{Ca}^{2+}]_i$ increase compared with the non-transfected control (data not shown). In addition, the pharmacological blockage of TRPC6 with SKF96365 (50 μM) or 2-APB (100 μM), nonselective TRPC channel blockers, significantly inhibited $[\text{Ca}^{2+}]_o$ - and the R-568-induced $[\text{Ca}^{2+}]_i$ increase, respectively (Fig. 3F and 3G). Taken together, these data indicated that TRPC6 is involved in the increase of $[\text{Ca}^{2+}]_i$ by CaSR activation.

Fig. 4. CaSR activation induces an increase in expression of active RhoA and it is dependent on TRPC6-dependent Ca²⁺ influx. (A) R-568 treatment induced a significant increase in active RhoA expression in podocytes. (**p*<0.01 vs. R-568, *n* = 3). (B) TRPC6 siRNA transfection reversed the increase in active RhoA induced by R-568 treatment. (***p*<0.01 vs. Ctrl, #*p*<0.05 vs. R-568, #*p*<0.05 vs. R-568 + Scr, *n*=3). +Scr: R-568+Scr, +TRPC6: R-568+TRPC6. (C) In the absence of extracellular Ca²⁺ (in the presence of 1 mM EGTA), the R-568-induced increase in RhoA activity was significantly reduced (*p*<0.05 vs. Ctrl, *n*=3).

TRPC6-dependent Ca²⁺ influx are required for RhoA activation induced by CaSR stimulation

RhoA activity was directly assessed by an affinity pull-down assay using the GST fusion protein rhotekin, which recognizes only the active form of RhoA (GTP-RhoA). As shown in Fig. 4A, treatment of podocytes with 10 nmol/L R-568 for 30 min caused a significant increase in RhoA activity compared with the control (*p*<0.01; *n*=3). The effect may be attributed to R-568-induced TRPC6 activation because transfection with TRPC6 siRNA attenuated the R-568-induced increase in RhoA activity (Fig. 4B), compared with cells transfected with scrambled siRNA (*p*<0.001; *n*=3). The transfection of scrambled siRNA did not affect RhoA activity. To further assess the contribution of Ca²⁺ influx in the increase in RhoA activity induced by CaSR activation, extracellular Ca²⁺ was depleted with 1 mM EGTA. As shown in Fig. 4C, the R-568-induced increase in RhoA activity was significantly reduced in the absence of extracellular Ca²⁺ (*p*<0.01; *n*=3). These findings indicated that upon activation of CaSR, the TRPC6-dependent Ca²⁺ influx contributes to RhoA activation.

TRPC6 regulates stress fiber formation and focal adhesions via RhoA in response to CaSR stimulation

To evaluate the functional role of TRPC6 on the CaSR activation-induced actin cytoskeleton, podocytes were stained with FITC-phalloidin. Because focal adhesions are required for the formation of stress fibers, focal adhesions were assessed by anti-vinculin immunostaining. In line with previous findings, treatment of podocytes with 10 nmol/L R-568 for 30 min significantly increased the numbers of stress fibers by approximately 32% compared with controls (Fig. 5A). A targeted siRNA approach to knockdown of RhoA was used to evaluate the effects of RhoA activity on changes in the actin cytoskeleton induced by R-568. The efficiency of RhoA-siRNA was confirmed by western blot analyses (Fig. 5B). As shown in Fig. 5C, transfection with RhoA-siRNA significantly abolished the increase in stress fibers and focal adhesions induced by R-568 compared with transfection with scrambled siRNA (*p*<0.001, *n*=3). As expected, transfection with TRPC6-siRNA also attenuated the increase in stress fibers and focal adhesions induced by R-568 (Fig. 5D, *p*<0.001, *n*=3). These findings suggested that TRPC6-mediated RhoA activation is associated with the changes in the actin cytoskeleton induced by CaSR stimulation.

Fig. 5. The role of TRPC6 and RhoA in the formation of stress fibers and focal adhesions induced by CaSR activation. Podocytes were double-label stained with FITC-phalloidin for stress fibers (green) and with vinculin antibodies (red). (A) R-568 treatment enhanced the formation of stress fibers and focal adhesions. Left, representative image; right, quantification evaluation. $**p < 0.01$ vs. Ctrl. (B) Western blot experiments showed that transfection with RhoA siRNA reduced RhoA protein expression ($***p < 0.001$ vs. Scr, $n = 3$). (C) RhoA siRNA transfection reversed the formation of stress fibers and focal adhesions induced by R-568 treatment. Left, representative image; right, quantification evaluation. $***p < 0.001$ vs. R-568+Scr. (D) TRPC6 knockdown also abolished the changes in actin cytoskeleton by R-568 treatment. Left, representative image; right, quantification evaluation. $***p < 0.001$ vs. R-568+Scr. Number of trials = 3; more than 50 cells were selected in each trial.

and focal adhesions induced by R-568 treatment. Left, representative image; right, quantification evaluation. $***p < 0.001$ vs. R-568+Scr. (D) TRPC6 knockdown also abolished the changes in actin cytoskeleton by R-568 treatment. Left, representative image; right, quantification evaluation. $***p < 0.001$ vs. R-568+Scr. Number of trials = 3; more than 50 cells were selected in each trial.

Discussion

Podocytes are highly differentiated epithelial cells that form the final barrier to protein loss. The podocyte foot processes form specialized cell junctions known as slit diaphragms, which are dynamically modified by actin cytoskeleton. Podocyte dysfunction and actin cytoskeleton disorganization lead to foot process effacement, disruption of slit diaphragms and proteinuria [19, 23-26]. Recently, several interesting studies have revealed that CaSR agonists, which are widely used to treat secondary hyperparathyroidism in patients with end-stage renal failure, have direct renoprotective actions [8, 9]. The direct protective effects of CaSR agonists may be mediated through actions on the actin cytoskeleton because R-568 increases actin stress fiber formation and focal adhesions and improves actin disorganization induced by puromycin aminonucleoside [9]. An investigation of the underlying mechanisms will provide a new treatment strategy for chronic kidney disease. In the present study, we

demonstrate that CaSR activation induces TRPC6-dependent Ca^{2+} influx, which mediates actin stress fiber formation induced by CaSR agonist via the RhoA pathway. This mechanism may explain the direct protective action of CaSR agonists.

Although downstream effects can be highly varied, the first reactions following CaSR activation are common; the stimulation of CaSR evokes an increase in $[\text{Ca}^{2+}]_i$ [1, 10]. However, the source of the $[\text{Ca}^{2+}]_i$ increase is cellular context-dependent. In this study, we show that CaSR stimulation in human podocytes induces not only Ca^{2+} release from the Ca^{2+} stores but also TRPC6-dependent Ca^{2+} entry. As shown in Fig. 2, both high $[\text{Ca}^{2+}]_o$ and R-568 induced an increase in $[\text{Ca}^{2+}]_i$ that included an initial rapid increase, which may represent intracellular Ca^{2+} mobilization, followed by a sustained increase, which may represent Ca^{2+} entry. Indeed, our experiments indicate that TRPC6 is at least partly responsible for the Ca^{2+} entry induced by CaSR stimulation because it is significantly abolished by both the pharmacological blockage of TRPC6 and the knockdown of TRPC6. CaSR is a group C GPCR that interacts with PLC through $G_{q/11}$ subunits of heterotrimeric G proteins, and therefore, its activation produces IP_3 and DAG. It is now widely accepted that TRPC6 can function as DAG-activated channels associated with the N-terminus (3-56 amino acids) of the TRPC6 protein [27, 28]. Therefore, it is likely that TRPC6 can be activated by the PLC-dependent production of DAG in response to CaSR stimulation. Our study also observed that the PLC inhibitor almost completely abolished the R-568-induced $[\text{Ca}^{2+}]_i$ increase. Compelling evidence has revealed that in colonic epithelial cells, CaSR stimulation by 5 mM $[\text{Ca}^{2+}]_o$ triggers DAG production [29], and in vascular smooth muscle cells, the upregulated TRPC6 mediated by CaSR is functionally sensitive to DAG [12]. DAG can directly increase TRPC6 activity and does not seem to affect the plasma membrane expression of TRPC6 [27, 28]. It is unclear whether CaSR stimulation in podocytes increases the plasma membrane expression of TRPC6 through another mechanism, and this topic warrants further investigation. Although group C GPCRs are molecularly distinct, their pharmacological profiles overlap, and they are constitutive dimers (either homo or hetero) [30]. The heterodimerization of CaSR with group I metabotropic glutamate receptors (mGluR1) in neurons has been established [31]. Interestingly, mGluR1 is also expressed in podocytes and may regulate actin cytoskeleton organization [32, 33]. Our unpublished data suggest that TRPC6 is involved in intracellular calcium signals induced by mGluR activation. Whether there is heterodimerization of CaSR with mGluR1 in podocyte and what its functional role is will be a valuable topic to investigate. Although voltage-gated calcium channels are not expressed in podocytes, [23] and TRPC6 is predominant among the Ca^{2+} permeable channels [16], other TRPC channels, such as TRPC5, and the ionotropic N-methyl-D-aspartate (NMDA) receptor also occur [34]. Therefore, this study cannot rule out the involvement of other calcium channels in the CaSR activation-induced Ca^{2+} influx.

Consistent with a previous study [9], our results demonstrate that CaSR stimulation with R-568 produces an increase in RhoA activity in podocytes. Interestingly, our study showed that the increased RhoA activity can be inhibited by transfection of TRPC6 siRNA, suggesting TRPC6-dependent Ca^{2+} entry is required for the activation of RhoA upon CaSR stimulation. CaSR stimulation inducing the activation of RhoA has been reported in several cell types, such as HEK-293 cells [35], Madin-Darby canine kidney (MDCK) cells [36], esophageal epithelial cells [37], renal collecting duct cells [38] and colonic epithelial cells [29]. However, the underlying mechanisms are unclear and may be highly dependent on cell type. In MDCK cells, CaSR stimulation induces RhoA activation via a $G_{12/13}$ signal pathway [36], whereas in HEK-293 cells, this occurs via a $G_{q/11}$ signal pathway [35]. In HEK-293 cells, the activation of G_q coupled to CaSR induces the interaction between the C terminal of CaSR and rho-guanine nucleotide exchange factors, which mediate RhoA activity [35]. Thus, there is a strong possibility that the activated G_q protein contributes to TRPC6 activation via the PLC pathway. Indeed, other GPCRs mediating RhoA activity via the G_q signaling pathway have widely been reported; however, relatively little is known about the involvement of TRPC6 in the activation of RhoA. Upon thrombin stimulation, TRPC6-dependent Ca^{2+} entry, but with neither other store-operated Ca^{2+} entry nor the release of calcium stores, is required

for RhoA activation and the consequent stress fiber formation in endothelial cells [39]. In podocytes, TRPC6 is also responsible for RhoA activation and stress fiber formation induced by angiotensin II stimulation [16].

It has been well accepted that RhoA promotes the formation of stress fibers and focal adhesion, producing a stationary podocyte phenotype, interpreted as a stable foot process, whereas the activity of Cdc42/Rac1 underlies the motile phenotype [19]. In line with this concept and a previous study, our study reveals that the inhibition of RhoA activity abolishes the formation of stress fibers and focal adhesions mediated by CaSR activation. Given the role of TRPC6 on the activation of RhoA mediated by CaSR stimulation in podocytes, it is not surprising that the blockage of TRPC6 abolishes the formation of stress fibers mediated by CaSR stimulation. This may indicate that TRPC6 is involved in the regulation of the actin cytoskeleton via the RhoA signaling pathway in response to CaSR stimulation. It remains unclear how the TRPC6 signal and the CaSR signal of podocytes are integrated for the CaSR stimulation. It has been demonstrated that, in podocytes, TRPC6 is localized in the lipid raft domain at plasma membranes [40] and TRPC6 is in a molecular complex with RhoA [16]. In some cell types, CaSR is distributed in a caveolin-rich plasma membrane domain [1]; however, this distribution does not seem to occur in podocytes [9]. This distribution requires further investigation.

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Disclosure Statement

The authors declare that no Disclosure Statement exists.

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