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Conditionally Immortalized Human Proximal-Tubular Epithelial Cells isolated from the urine of a healthy subject express functional Calcium Sensing Receptor

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Running Head: ciPTEC express functional CaSR

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Abstract

The calcium-sensing receptor (CaSR) is a G protein coupled receptor, which plays an essential role in regulating Ca^{2+} homeostasis. Here we show that conditionally immortalized proximal tubular epithelial cell line (ciPTEC) obtained by immortalizing and subcloning cells exfoliated in the urine of a healthy subject expresses functional endogenous CaSR.

ciPTE cells expressed ZO-1 protein, aquaporin 1 and NHE3 thus confirming their epithelial and proximal tubule origin respectively. Immunolocalization studies of polarized ciPTEC grown on filters revealed the apical localization of the receptor. By Western blotting of ciPTEC lysates, both forms of CaSR at 130 and ~200kDa were detected corresponding to the monomeric and mature receptor. Of note, functional studies indicated that both external calcium and the positive CaSR allosteric modulator NPS-R568, induced a significant increase in cytosolic calcium, proving a high sensitivity of the endogenous receptor to its agonists. Cytosolic calcium levels were 46.2±2.22% (vs ATP 100%) after stimulation with 2.5µM Ca^{2+} and 37±1.76% (vs ATP 100%) after stimulation with 2.5µM NPS-R568. Calcium depletion from the ER using CPA (cyclopiazonic acid) abolished the increase in cytosolic calcium elicited by NPS-R568 confirming calcium exit from intracellular stores. Activation of CaSR by NPS-R significantly reduced the increase in cAMP elicited by forskolin, a direct activator of adenylatecyclase, thus confirming the functional expression of the receptor in this cell line.

CaSR expressed in ciPTEC was found to interact with G_q as downstream effector which in turn can cause release of calcium from intracellular stores via PLC activation. We conclude that human proximal tubular ciPTEC express functional CaSR and respond to its activation with a release of calcium from intracellular stores. These cell lines represent a valuable tool for research into the disorder associated with gain or loss of function of the CaSR by producing cell lines from patients.

Keywords

conditionally immortalized proximal tubular epithelial cell line, ciPTEC; calcium sensing receptor, CaSR; NPS-R568, calcium signaling

Introduction

The extracellular calcium-sensing receptor (CaSR) is a G protein coupled receptor, originally cloned from the bovine parathyroid gland and successively identified in various organs (6, 7, 45). Besides the parathyroid gland, the key CaSR expressing organs are intestine, bone, and kidney (43, 45). The CaSR senses changes in extracellular calcium concentrations and regulates parathyroid hormone (PTH) secretion and renal tubular calcium reabsorption to maintain serum calcium levels within the normal range. Ligand binding to the CaSR results in conformational changes of the intracellular loops, G protein-dependent stimulation of phospholipase C causing an accumulation of inositol 1,4,5-trisphosphate and rapid release of calcium ions from intracellular stores. The increase in intracellular calcium results in activation of protein kinase C and CaSR also activates the mitogen-activated protein kinase (MAPK) pathway (4, 34, 36).

A significant limitation in studies of CaSR function in the kidney has arisen from the difficulty of clearly defining the patterns of CaSR expression along the nephron. Although all studies agree that the CaSR is expressed along the basolateral membrane of the thick ascending limb of Henle (TAL), a key site of regulated calcium reabsorption (24, 35, 37), different groups have reached different conclusions regarding the nature and level of CaSR expression in other sites. Along the nephron, Riccardi et al (37) showed a specific receptor localization on the apical surface of the proximal tubule (PT). In contrast, Loupy et al (24) reported that CaSR expression within the kidney is restricted to the TAL. A possible explanation for this discrepacies may be due to the use of commercial antibodies against specific fragments of the receptor. Riccardi and colleagues (8, 37,

38), using polyclonal antibodies against both full-length CaSR and specific regions of the exofacial domains coupled with antigen retrieval, were successful in obtaining a clear apical staining of the CaSR at the base of the brush border of PT.

In the kidney, the CaSR performs different tasks depending on the various tubular segments in which it is located (3, 20, 34, 44). In the PT, CaSR located on the apical membrane senses the increase in calcium luminal concentrations and inhibits cAMP production induced by PTH. In proximal tubules, PTH causes phosphate excretion by internalization and degradation of phosphate reabsorption carriers in subapical vesicles derived from brush border (1, 48). In the TAL, activation of CaSR expressed on the basolateral membrane (35) inhibits calcium paracellular reabsorption by blocking claudin-16 by activation of claudin-14 transcription (15). In the cortical distal convoluted tubule, CaSR is expressed on the basolateral membrane, where it reduces calcium active reabsorption inhibiting calcium pump activity (PMCA) (2, 13). In the collecting duct, CaSR is expressed on the apical membrane of the principal and intercalated cells (35, 45) where, during acute vasopressin action, its activation reduces AQP2 mediated water reabsorption and urinary concentration (31). CaSR has been shown to reduce also the vasopressin-induced AQP2 expression, via a calmodulin-dependent mechanism (9).

Regarding the physiological role of CaSR expressed in the PT, recent interesting studies performed in rats and *in vitro* perfused mouse proximal tubules, demonstrated a role of the CaSR in enhancing proximal tubular fluid absorption and urinary acidification by stimulation of luminal Na^+/H^+ exchanger (NHE) activity (10, 49). Activation of NHE will favor the ionization of calcium so that the ionized calcium is delivered to the distal portions of the nephron where it would be more easily reabsorbed, thus avoiding calcium precipitation along more distal segments of the nephron. Therefore the new concept is that CaSR expressed in the PT would play an active role in modulating

PT fluid absorption as well as acid secretion and postulated its possible involvement in prevention of renal stone disease.

Based on these recent evidence it would be interesting to clarify at cellular level the molecular mechanism occurring in PT epithelial cells activated by increased luminal calcium concentration and leading to enhanced fluid reabsorption in the proximal tubule, a process related to activation of CaSR. To this end, a cell line which would be eligible is represented by the conditionally immortalized human proximal tubular epithelial cells (ciPTEC), whose isolation from urine and immortalization has been developed by Wilmer and coworkers (53). In recent studies, ciPTEC derived from patients were used to demonstrate the defect in Na-dependent phosphate reabsorption (52) in cystinosis and altered receptor-mediated endocytosis and the mechanisms of endosomal acidification occurring in (16). These cell lines therefore may represent a valuable tool for research into the disorder associated with CaSR mutations by producing cell lines from patients. ciPTEC can be maintained for at least 45 passages and present PT characteristics when cultured at the non-permissive temperature of 37°C for 10 days (53).

We provide here the first evidence that ciPTEC endogenously express CaSR at protein level. Moreover, exposure of ciPTEC to clinically relevant concentrations of calcium or to the positive allosteric CaSR modulator NPS-R568 resulted in an increase in intracellular calcium and in a decrease of cytosolic cAMP levels, demonstrating the expression of a functional receptor. ciPTEC could aid in understanding the role of CaSR in proton secretion and fluid reabsorption in proximal tubules.

Materials and Methods

Materials

All chemicals were purchased from Sigma (Sigma-Aldrich, Milan, Italy). Fura-2AM was obtained from Molecular Probes (Life Technologies, Monza, Italy). NPS-R568 was kindly gifted by Amgen (Amgen Dompé S.p.a., Milan, Italy). Media for cell culture were from Lonza (Lonza s.r.l., Milan, Italy).

Antibodies

Monoclonal CaSR antibody recognizing amino-acid 15–29 at the extracellular N-terminus (17, 47) was from Sigma-Aldrich, Milan, Italy. To detect the expression of AQP2, we used antibodies against the 20-amino acid residue segment just N-terminal from the polyphosphorylated region of rat AQP2 (CLKGLEPDTDWEEREVRRRQ) (19, 41). Mouse monoclonal Na^+/H^+ Exchanger-3 (NHE3) and rabbit anti $Na^+/K^+/Cl^-$ (NKCC2) cotransporter antibodies were from Chemicon (Millipore, Merck S.p.A., Milan, Italy). Rabbit polyclonal anti-AQP1, mouse anti-ZO-1 and mouse anti-G_q antibodies were obtained from Santa Cruz Biotechnologies (TebuBio, Milan, Italy). Secondary goat anti-mouse-488 conjugate antibody and streptavidin-488 conjugate were from Alexa Fluor (Molecular Probes, Eugene, Oregon, USA). Secondary rabbit anti-mouse-biotin, goat anti-rabbit and goat anti-mouse antibodies were purchased from Sigma-Aldrich, Milan, Italy.

PTE Cell Line Generation

Primary cells were cultured as described by Wilmer and coworkers (51) by collecting mid-stream urine. The cell line used in this study derived from a female healthy donor, born in 1989, who was 12 years old when her urine was taken. A parental consent was obtained.

Urine was centrifuged (223×g, 5min, room temperature) within 5h after collection. After washing in phosphate-buffered saline (PBS) and a second centrifugation step, urine sediment was resuspended in 3ml PTEC culture medium (DMEM Ham's F12) supplemented with 10% fetal bovine serum (FBS), 100i.u./ml penicillin, 100mg/ml streptomycin, ITS (5μg/ml insulin, 5μg/ml transferrin and 5ng/ml selenium), 36ng/ml hydrocortisone, 10ng/ml epidermal growth factor (EGF) and 40pg/mltriiodothyronine (11). The suspension was transferred to a 25-cm^2 tissue culture flask and placed at 37°Cin a 5% CO_2 incubator. The medium was refreshed every 2–3 days(53).

Immortalization, subcloning and generation of ciPTEC

Primary cells were immortalized as described (53). Briefly, cells were infected with SV40T and hTERT vectors using the amphotropic packaging cell line PA 317 (39), containing respectively geneticin (G418) and hygromycin resistance (30, 40). Subconfluent cell layers were transferred to 33°C and selected by using G418 (400μg/ml) and hygromycin B (25μg/ml) for 10 days. After being cultured for 2 weeks at 33°C, single cell clones were visible and picked by using cloning discs drained in trypsin/EDTA. For the following experiments, cells were cultured at 33°C to 70% confluency, followed by maturation for 10 days at 37°C during which the cells formed a confluent monolayer. Experimental procedures were performed on the cloned cells between passages 15 and 40. Morphology of ciPTEC was evaluated by using phase contrast microscopy.

Immunofluorescence Microscopy

Immunofluorescence localization of CaSR in polarized ciPTEC was performed as previously described (22). ciPTEC were cultured on polyester Transwell inserts and after 10 days of maturation at 37°C, were fixed using 2% (w/v) paraformaldehyde in HBSS supplemented with 2% (w/v) sucrose for 5 min and permeabilized in 0.3% (v/v) triton X-100 in HBSS for 10 min.

Cells were incubated with antibodies diluted in block solution containing 2% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) tween-20 in HBSS against the tight junction protein zonula occludens 1 (ZO-1, 1:200 dilution) and the calcium-sensing receptor (CaSR, 1:800 dilution) at 4°C overnight. Following treatment with secondary goat-anti-mouse-Alexa488 conjugate for ZO-1 and rabbit-anti-mouse-biotin antibody followed by Streptavidin-488 for CaSR, samples were mounted on glass slides with Mowiol. Images were obtained with a confocal microscope Leica TCS SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Cell Preparations

ciPTEC were seeded onto Ø60 mm dishes and grown at 37°C for 10 days, then were lysed in Cell Fractionation Buffer (20mM NaCl, 130mM KCl, 1mM $MgCl_2$, 10mM Hepes, pH 7.5) in the presence of proteases (1mM PMSF, 2mg/ml leupeptin and 2mg/ml pepstatin A) and phosphatases (10mMNaF and 1mM sodium orthovanadate) inhibitors. Cellular debris was removed by centrifugation at 12,000×g for 20min at 4°C. The supernatants were collected and used for immunoblotting studies.

Immunoprecipitation

For immunoprecipitation experiments, ciPTEC were seeded onto Ø100mmdishes and grown at 37°C for 10 days. IP experiments were performed as described (23, 42). Briefly, ciPTEC were lysed with 1% Triton X-100, 150mMNaCl, 25mM Hepes (pH 7.4) in the presence of protease inhibitors (1mM PMSF, 2mg/ml leupeptin and 2mg/ml pepstatinA). The supernatants were precleared with 50µl of immobilized protein-A and incubated O/N with anti-$G_{\alpha q}$ antibodies coupled to protein A-sepharose. As negative control, lysates were incubated with non-specific rabbit IgG. The

immunocomplexes were washed three times, resuspended in50µl of Laemmli buffer and subjected to immunoblotting using CaSR antibodies.

Gel Electrophoresis and Immunoblotting

ciPTEC lysates were separated on 13% bis-tris acrylamide gels under reducing conditions. Protein bands were electrophoretically transferred onto Immobilon-P membranes (Millipore Corporate Headquarters, Billerica, USA) for Western blot analysis, blocked inTBS-Tween-20 containing 3% BSA and incubated with primary antibodies O/N. Immunoreactive bands were detected with secondary antibody conjugated to horseradish peroxidase (HRP) obtained from SantaCruz Biotechnologies (Tebu-Bio, Milan, Italy). Membranes were developed using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) with Chemidoc System (Bio-Rad Laboratories, Milan, Italy).

Video-Imaging Experiments

ciPTEC were grown onØ40mm glass coverslips at 37°C for 10 days, then were loaded with 4µMFura-2AM for 15min at 37°C in DMEM. Ringer's Solution was used to perfuse cells during the experiment containing 120mMNaCl, 4mM KCl, 15mM $NaHCO_3$, 1mM $MgCl_2$, 15mM Hepes, 0.5mM NaH_2PO_4, 10mM Glucose, 1mM $CaCl_2$, 0.5mM Na_2HPO_4, 0.4mM $MgSO_4$, pH 7.4[modified by(25, 26)]. In fluorescence measurements, the coverslips with dye-loaded cells were mounted in a perfusion chamber (FCS2 Closed Chamber System, BIOPTECHS, Butler, U.S.A.) and measurements were performed using an inverted microscope (Nikon Eclipse TE2000-S microscope) equipped for single cell fluorescence measurements and imaging analysis. The sample was illuminated through a 40X oil immersion objective (NA=1.30). The Fura-2AM loaded sample was excited at 340 and380nm. Emitted fluorescence was passed through a dichroic mirror, filtered at 510nm (Omega Optical,

Brattleboro, VT,USA) and captured by a cooled CCD camera (Cool SNAP HQ,Photometrics). Fluorescence measurements were carried out using Metafluor software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). The ratio of fluorescence intensities at340 and 380nm was plotted.

Fluorescence Resonance Energy Transfer (FRET) Measurements

For evaluation of cAMP levels in ciPTEC, FRET experiments were performed as described (41). Briefly, ciPTEC were seeded onto Ø20mm glass coverslips at 37°C for 10 days and transiently transfected with a plasmid (0.4μg of DNA/cm^2) encoding the H96 probe containing cAMP binding sequence of Epac1 between CFP and cp^{173}Venus-Venus (gift from dr. K. Jalink) (46), using lipofectamine (1 μg/μl) according to the protocol provided by the manufacturer (Life Technologies, Monza Italy). Experiments were performed 48 hours post-transfection.

After overnight treatment with indomethacin ($5x10^{-5}$ M), a prostaglandin synthesis inhibitor that reduced basal cAMP, cells were left under basal condition or stimulated with forskolin (10^{-5} M for 5 min) or alternatively with NPS-R568 (10 μM for 30 min or 20 μM for 15 min), left unstimulated or stimulated with forskolin (10^{-5} M for 5 min). All treatments were performed in Ringer's Solution described above, containing 1.5mM $CaCl_2$.

FRET measurements were carried out using MetaMorph software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). Specifically binding of cAMP to the Epac1 results in an intramolecular steric conformational modification causing an increase in the distance between the donor (CFP) and the acceptor (Venus) and a decrease of FRET process. CFP and Venus were excited at 430 and 480 nm, respectively; fluorescence emitted from CFP and Venus was measured at 480/30 and 545/35 nm, respectively. FRET from CFP to Venus was determined by excitation of CFP and

measurement of fluorescence emitted from Venus. Corrected normalized FRET values were determined accordingly to Tamma (41).

Results

Characterization of conditionally immortalized Proximal Tubular Epithelial Cells (ciPTEC)

Since a mixture of cell types can be exfoliated in urine, a characterization study was performed after subcloning the cell line to confirm its PT origin (12). At 37°C, cell monolayers of the subclones expressed ZO-1 protein, indicating the epithelial origin of cells with development of tight junctions (Fig. 1). The presence of PT-specific proteins AQP1 and NHE3 was demonstrated by immunoblotting of cells cultured for 10 days at 37°C (Fig. 2A-B) (29). Contamination of cells originating from other segments known to express CaSR were excluded by immunoblotting experiments performed using Na^+,K^+,$2Cl^-$ cotransporter (NKCC2) and AQP2 as markers of the TAL and the collecting duct respectively. While both forms of NKCC2 at 250 and 160kDa, corresponding to the mature and the monomeric form (Fig. 2C), and AQP2 (Fig. 2D) were detected in mice kidney homogenate, medulla and cortex, used as positive controls, no expression of these transporters was detected in ciPTEC lysates, confirming the purity of this cell line.

Endogenous CaSR expression in ciPTEC

We next evaluated the CaSR expression in ciPTEC by western blotting. Both forms at 130 and ~200kDa, corresponding to the monomeric and mature CaSR receptor, were detected in ciPTEC clones grown at 37°C for 10 days as well as in HEK293 cells transfected with human CaSR, used as positive control (Fig. 3A). A very low expression was found in ciPTEC lysates obtained from less differentiated cells grown at 33°C (data not shown).

Immunofluorescence CaSR localization in subconfluent monolayer of polarized ciPTEC performed after 10 days of maturation at 37°C showed a plasma membrane expression of the receptor (Fig. 3B).

ciPTEC express functional CaSR

To evaluate whether the immunodetected CaSR protein is a functional receptor, ciPTEC were loaded with Fura-2AM (4μM) and exposed to raising concentrations of the CaSR physiological agonist, specifically to 2mM, 5mM and 10mM of extracellular calcium ($[Ca^{2+}]_o$). Variations in intracellular calcium (Ca^{2+}_i) were evaluated by single-cell epifluorescence imaging. Figure 4A shows a representative time course of fluorescence responses. CaSR stimulation caused an increase in Ca^{2+} already at 2mM $[Ca^{2+}]_o$ consistent with its functional expression, proving a high sensitivity of the endogenous receptor to low concentrations of its agonists. For a more specific functional analysis, cells were treated with 10μM NPS-R568, the positive allosteric CaSR modulator that increases the sensitivity of the receptor for calcium (27). This compound is known to act selectively on the CaSR (28). Under these conditions, a bell-shaped calcium-response was observed (Fig. 4B). Statistical analysis of the fluorescence responses revealed that cytosolic calcium levels were 46.32±2.28% (vs ATP 100%, n=23) after stimulation with 2.5μM Ca^{2+} and 37.48±1.71% (vs ATP 100%, n=25) after stimulation with 10μM NPS-R568. The NPS-R568 effect was stereo selective. In fact, experiments using the *S* enantiomer which is 10- to 100-fold less potent than the *R* enantiomers (28) showed that in cells treated with NPS-S568 (10μM) the increase in cytosolic calcium was negligible (4.21±0.54% vs ATP 100%, n=7, Fig. 4C).

To investigate the origin of the calcium release upon specific CaSR activation with NPS-R568, calcium was depleted from the ER using 40μM CPA (cyclopiazonic acid), an inhibitor of the Sarco-Endoplasmatic Reticulum Calcium ATPasi (SERCA). Under this experimental condition the increase

in cytosolic calcium elicited by NPS-R568 was abolished, confirming that activation of CaSR results in calcium release from intracellular stores, likely the ER (Fig. 5).

As discussed, in PT the CaSR expressed on the luminal membrane senses the increase in luminal calcium concentrations and inhibits cAMP production induced by PTH (35). To further evaluate CaSR signaling in ciPTEC, cAMP levels were measured by FRET experiments using the H96 probe containing cAMP binding sequence of Epac1 between CFP and Venus. Cells were treated with 10μM Forskolin (FK), a direct activator of adenylate cyclase, for 5 min, with the positive allosteric CaSR modulator NPS-R568 10μM for 30 min or 20μM for 15 min, and with both NPS-R568 10μM or 20μM and FK 10μM in the last 5 min. CaSR activation with NPS-R significantly reduced FK-induced cAMP increase (Fig.6). No changes in cAMP levels were detected in NPS-R568 10μM or 20μM treated cells with respect to cells left under basal condition (CTRL).

CaSR expressed in ciPTEC interact with G_q

The extracellular calcium-sensing receptor belongs to the C family of the G-protein-coupled receptors (GPCR) and has been shown to couple to G_q in several tissues (5, 6, 18, 50). To evaluate whether CaSR expressed in ciPTEC is functionally coupled with *G_q*, co-immunoprecipitation studies were performed. ciPTEC lysates were immunoprecipitated with specific anti-G_q antibodies and immunoprecipitates were next probed with anti-CaSR antibodies revealing positive CaSR bands in all conditions predominantly corresponding to the mature forms of the receptor CaSR at 160kDa and ~200kDa. These studies indicate that CaSR couple to with G_q as downstream effector in ciPTEC (Fig. 7).

Discussion

In this study, we provide the first evidence that conditionally immortalized human proximal tubular epithelial cells, ciPTEC, isolated from urine of a healthy volunteer, endogenously express a functional CaSR.

The immortalization of non-invasively collected cells developed by Wilmer and coworkers has enabled the production of human cells maintaining PT characteristics and proliferating for at least 45 passages (53). For the culture of renal cells from urine, Wilmer and co-workers have used the immortalization methodology by using SV40T, originally described by Racusen and colleagues (32). The authors have cultured conditionally immortalized PTEC clones from the urine of two patients with nephropathic cystinosis, an inherited disorder of PT transport attributable to lysosomal cystine accumulation. The detailed characterization of the ciPTEC including their viability, proliferation capacity, formation of a tight monolayer and expression of multiple PT specific endogenous organic ion transporters, indicates the feasibility of using urinary cells as a source for obtaining human renal material for in vitro research by collecting cells from the urine of patients (53). Moreover, the expression pattern and the function of the majority of PT transporters was similar between cells isolated from urine compared to those isolated from kidney tissue and immortalized using the same methodology (22).

In a very recent work, ciPTEC established from three patients affected by human Dent disease have been crucial for understanding the molecular defect in renal reabsorption, demonstrating that CLC-5 mutations have multiple effects on endosomal acidification and receptor-mediated endocytosis (14).

The major goal of the present contribution was to evaluate and characterize ciPTEC for the functional expression of the CaSR. The availability of a human source of cells from patients affected by mutations of this receptor might be of great relevance for confirming, using a non-invasive ex

vivo approach, our recent in vitro data explaining the molecular basis of gain of function variants of CaSR (33). Specifically we have shown that renal cells expressing gain of function CaSR variants showed a significant increase in SERCA activity and expression and a reduced PMCA expression. This combined parallel regulation increases the ER to cytosol calcium gradient explaining the higher sensitivity of CaSR gain-of-function variants to external calcium leading to an exacerbate signaling (33).

In the aim of using ciPTEC obtained by immortalizing and subcloning cells exfoliated in the urine of an healthy subject as possible model systems for the study of human renal disease associated with CaSR mutations, in the present contribution we characterized ciPTEC cells for functional CaSR expression. As a first step, cells obtained after subcloning were screened for the expression of the zona occludens 1 protein (ZO-1), of the AQP1 and Na^+/H^+ exchanger-3 (NHE3) proteins, proving their epithelial and proximal tubule origin respectively. The pure PT derivation of this cell line was demonstrated by the absence of other tubular segments proteins expression such as NKCC2 and AQP2, markers of the TAL and the collecting duct respectively.

Isolated ciPTEC expressed both the monomeric and the mature glycosylated form CaSR receptor that localized at the apical plasma membrane. Of note, functional experiments showed that activation of CaSR expressed in ciPTEC, obtained by either raising the luminal calcium ion concentration or by the specific CaSR positive allosteric activator NPS-R568 (28), caused a release of calcium from an intracellular store likely corresponding to the ER. Functional expression of CaSR in ciPTEC was further confirmed by its ability to reduce FK-induced increase in cAMP as it occurs in PT under PTH action.

In this work we also evaluated G protein signaling associated to CaSR expressed in ciPTEC. The reported data obtained by co-immunoprecipitation studies identified G_q as one of the possible downstream effectors of CaSR in ciPTEC.

It is known that CaSR can couple to several G proteins such as *a*. $G_{q/11}$, which stimulates phospholipase C (PLC) thereby producing diacylglycerol and inositol 1,4,5-trisphosphate (IP3) causing releases of calcium from intracellular stores, *b*. Gi, resulting in adenylate cyclase inhibition, *c*. $G_{12/13}$ causing activation of Rho kinase (21, 34). In addition to inhibiting adenylate cyclase via Gi, the CaSR can also reduce cAMP indirectly by reducing the activity of calcium sensitive adenylate cyclase or activating phosphodiesterase (14). Our data obtained in ciPTEC indicate that the activation of CaSR expressed in proximal tubule causes increase in intracellular calcium due to CaSR coupling to G_q resulting in PLC activation and IP3-dependent release in intracellular calcium. By demonstrating that human renal proximal tubular cells ciPTEC harbor endogenous and functional CaSR, our study provides a human cell model relevant for research into the disorder associated with gain or loss of function mutations of the CaSR by producing cell lines from patients.

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Figure legends

Figure 1: Expression of epithelial marker ZO-1. Confluent ciPTEC monolayers were analyzed after 10 days of maturation at 37°C. Positive staining indicates the formation of tight junctions.

Figure 2: (A-B) Expression of the proximal tubule specific proteins. Western blotting of AQP1 (A) and NHE3 (B) in homogenates of ciPTEC clones, after 10 days of maturation at 37°C. AQP1 and NHE3 expression in human kidney homogenate were used as positive controls. (C-D) Expression of proteins markers of the TAL and the collecting duct in ciPTEC. NKCC2 (C) and AQP2 (D) were not detected in ciPTEC homogenates confirming the absence of contamination of cells originating from other segments. Westrn blotting of mice kidney homogenate, medulla and cortex, are shown as positive controls.

Figure 3: Expression and localization of CaSR. (A) Immunodetection of CaSR in homogenates of ciPTEC clones, after 10 days of maturation at 37°C. Specific anti-CaSR antibodies revealed both CaSR forms at 130 and ~200 kDa, corresponding to the monomeric and mature receptor, compared to CaSR expression in HEK293 lysates, transiently transfected with human CaSR-wt used as positive control. (B) Immunofluorescence localization of CaSR in polarized ciPTEC, showing its predominant apical plasma membrane localization.

Figure 4: Effects of increasing concentration of CaSR agonists on $[Ca^{2+}]_i$ levels. Cells were grown for 10 days at 37°C and stimulated with increasing levels of $[Ca^{2+}]_o$ (A) and NPS-R568 10μM (B). Cells were also treated with the much less potent stereoisomer NPS-S568 10μM (C), confirming the specific activation of CaSR elicited by NPS-R568(28). Fluorescence ratio 340/380nm was recorded. Each trace is representative of 3–4 different experiments with similar results.

Figure 5: Effect of ER calcium depletion on the response to NPS-R568 in ciPTEC. Calcium depletion from the ER using CPA (cyclopiazonic acid) abolished the increase in cytosolic calcium elicited by NPS-R568, confirming the origin of calcium exit from intracellular stores.

Figure 6: Evaluation of cAMP levels in PTEC by FRET analysis. Histograms compare changes of NFRET ratio under different experimental conditions. FK stimulation caused a significant ($P<0.0001$) increase in cAMP levels (depicted by a decrease in NFRET ratio). FK-induced increase in cAMP was significantly and proportionally reduced in the presence of NPS-R568 10μM and 20μM ($P<0.01$ and $P<0.0001$ respectively). NPS-R568 did not altered cAMP levels under basal conditions.

Figure 7: Co-Immunoprecipitation of G_q and CaSR proteins. ciPTEC clones homogenates were subjected to immunoprecipitation with specific antibody against G_q. Immunoprecipitates were probed with anti-CaSR antibody. CaSR and G_q expression in rat kidney homogenate is shown as positive control.

References

1. **Ba J and Friedman PA.** Calcium-sensing receptor regulation of renal mineral ion transport. *Cell calcium* 35: 229-237, 2004.
2. **Blankenship KA, Williams JJ, Lawrence MS, McLeish KR, Dean WL, and Arthur JM.** The calcium-sensing receptor regulates calcium absorption in MDCK cells by inhibition of PMCA. *American journal of physiology Renal physiology* 280: F815-822, 2001.
3. **Bozic M and Valdivielso JM.** Calcium signaling in renal tubular cells. *Advances in experimental medicine and biology* 740: 933-944, 2012.
4. **Brown EM.** Role of the calcium-sensing receptor in extracellular calcium homeostasis. *Best practice & research Clinical endocrinology & metabolism* 27: 333-343, 2013.
5. **Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, and Hebert SC.** Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 366: 575-580, 1993.
6. **Brown EM and MacLeod RJ.** Extracellular calcium sensing and extracellular calcium signaling. *Physiological reviews* 81: 239-297, 2001.
7. **Brown EM, Pollak M, Chou YH, Seidman CE, Seidman JG, and Hebert SC.** The cloning of extracellular Ca(2+)-sensing receptors from parathyroid and kidney: molecular mechanisms of extracellular Ca(2+)-sensing. *The Journal of nutrition* 125: 1965S-1970S, 1995.
8. **Brown EM, Pollak M, Riccardi D, and Hebert SC.** Cloning and characterization of an extracellular Ca(2+)-sensing receptor from parathyroid and kidney: new insights into the physiology and pathophysiology of calcium metabolism. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 9: 1703-1706, 1994.
9. **Bustamante M, Hasler U, Leroy V, de Seigneux S, Dimitrov M, Mordasini D, Rousselot M, Martin PY, and Feraille E.** Calcium-sensing receptor attenuates AVP-induced aquaporin-2 expression via a calmodulin-dependent mechanism. *Journal of the American Society of Nephrology : JASN* 19: 109-116, 2008.
10. **Capasso G, Geibel PJ, Damiano S, Jaeger P, Richards WG, and Geibel JP.** The calcium sensing receptor modulates fluid reabsorption and acid secretion in the proximal tubule. *Kidney international* 84: 277-284, 2013.
11. **Detrisac CJ, Sens MA, Garvin AJ, Spicer SS, and Sens DA.** Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney international* 25: 383-390, 1984.
12. **Dorrenhaus A, Muller JI, Golka K, Jedrusik P, Schulze H, and Follmann W.** Cultures of exfoliated epithelial cells from different locations of the human urinary tract and the renal tubular system. *Archives of toxicology* 74: 618-626, 2000.
13. **Gamba G and Friedman PA.** Thick ascending limb: the Na(+):K (+):2Cl (-) co-transporter, NKCC2, and the calcium-sensing receptor, CaSR. *Pflugers Archiv : European journal of physiology* 458: 61-76, 2009.
14. **Geibel JP and Hebert SC.** The functions and roles of the extracellular Ca2+-sensing receptor along the gastrointestinal tract. *Annual review of physiology* 71: 205-217, 2009.
15. **Gong Y, Renigunta V, Himmerkus N, Zhang J, Renigunta A, Bleich M, and Hou J.** Claudin-14 regulates renal Ca(+)(+) transport in response to CaSR signalling via a novel microRNA pathway. *The EMBO journal* 31: 1999-2012, 2012.
16. **Gorvin CM, Wilmer MJ, Piret SE, Harding B, van den Heuvel LP, Wrong O, Jat PS, Lippiat JD, Levtchenko EN, and Thakker RV.** Receptor-mediated endocytosis and endosomal acidification is impaired in proximal tubule epithelial cells of Dent disease patients. *Proceedings of the National Academy of Sciences of the United States of America* 110: 7014-7019, 2013.
17. **Handlogten ME, Huang C, Shiraishi N, Awata H, and Miller RT.** The Ca2+-sensing receptor activates cytosolic phospholipase A2 via a Gqalpha -dependent ERK-independent pathway. *The Journal of biological chemistry* 276: 13941-13948, 2001.
18. **Hofer AM and Brown EM.** Extracellular calcium sensing and signalling. *Nature reviews Molecular cell biology* 4: 530-538, 2003.

19. **Hoffert JD, Pisitkun T, Wang G, Shen RF, and Knepper MA.** Quantitative phosphoproteomics of vasopressin-sensitive renal cells: regulation of aquaporin-2 phosphorylation at two sites. *Proceedings of the National Academy of Sciences of the United States of America* 103: 7159-7164, 2006.
20. **Houillier P.** Calcium-sensing in the kidney. *Current opinion in nephrology and hypertension* 22: 566-571, 2013.
21. **Huang C and Miller RT.** The calcium-sensing receptor and its interacting proteins. *Journal of cellular and molecular medicine* 11: 923-934, 2007.
22. **Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, Bank RA, van den Heuvel LP, Hoenderop JG, and Masereeuw R.** A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. *Experimental cell research* 323: 87-99, 2014.
23. **Kamsteeg EJ, Hendriks G, Boone M, Konings IB, Oorschot V, van der Sluijs P, Klumperman J, and Deen PM.** Short-chain ubiquitination mediates the regulated endocytosis of the aquaporin-2 water channel. *Proceedings of the National Academy of Sciences of the United States of America* 103: 18344-18349, 2006.
24. **Loupy A, Ramakrishnan SK, Wootla B, Chambrey R, de la Faille R, Bourgeois S, Bruneval P, Mandet C, Christensen EI, Faure H, Cheval L, Laghmani K, Collet C, Eladari D, Dodd RH, Ruat M, and Houillier P.** PTH-independent regulation of blood calcium concentration by the calcium-sensing receptor. *The Journal of clinical investigation* 122: 3355-3367, 2012.
25. **Mekahli D, Sammels E, Luyten T, Welkenhuyzen K, van den Heuvel LP, Levtchenko EN, Gijsbers R, Bultynck G, Parys JB, De Smedt H, and Missiaen L.** Polycystin-1 and polycystin-2 are both required to amplify inositol-trisphosphate-induced Ca2+ release. *Cell calcium* 51: 452-458, 2012.
26. **Miyakawa A, Ibarra C, Malmersjo S, Aperia A, Wiklund P, and Uhlen P.** Intracellular calcium release modulates polycystin-2 trafficking. *BMC nephrology* 14: 34, 2013.
27. **Nemeth EF, Steffey ME, and Fox J.** The parathyroid calcium receptor: a novel therapeutic target for treating hyperparathyroidism. *Pediatric nephrology (Berlin, Germany)* 10: 275-279, 1996.
28. **Nemeth EF, Steffey ME, Hammerland LG, Hung BC, Van Wagenen BC, DelMar EG, and Balandrin MF.** Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proceedings of the National Academy of Sciences of the United States of America* 95: 4040-4045, 1998.
29. **Nielsen S, Kwon TH, Christensen BM, Promeneur D, Frokiaer J, and Marples D.** Physiology and pathophysiology of renal aquaporins. *Journal of the American Society of Nephrology : JASN* 10: 647-663, 1999.
30. **O'Hare MJ, Bond J, Clarke C, Takeuchi Y, Atherton AJ, Berry C, Moody J, Silver AR, Davies DC, Alsop AE, Neville AM, and Jat PS.** Conditional immortalization of freshly isolated human mammary fibroblasts and endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 98: 646-651, 2001.
31. **Procino G, Mastrofrancesco L, Tamma G, Lasorsa DR, Ranieri M, Stringini G, Emma F, Svelto M, and Valenti G.** Calcium-sensing receptor and aquaporin 2 interplay in hypercalciuria-associated renal concentrating defect in humans. An in vivo and in vitro study. *PloS one* 7: e33145, 2012.
32. **Racusen LC, Monteil C, Sgrignoli A, Lucskay M, Marouillat S, Rhim JG, and Morin JP.** Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines. *The Journal of laboratory and clinical medicine* 129: 318-329, 1997.
33. **Ranieri M, Tamma G, Di Mise A, Vezzoli G, Soldati L, Svelto M, and Valenti G.** Excessive Signal Transduction of Gain-of-Function Variants of the Calcium-Sensing Receptor (CaSR) Are Associated with Increased ER to Cytosol Calcium Gradient. *PloS one* 8: e79113, 2013.
34. **Riccardi D and Brown EM.** Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *American journal of physiology Renal physiology* 298: F485-499, 2010.
35. **Riccardi D, Hall AE, Chattopadhyay N, Xu JZ, Brown EM, and Hebert SC.** Localization of the extracellular Ca2+/polyvalent cation-sensing protein in rat kidney. *The American journal of physiology* 274: F611-622, 1998.
36. **Riccardi D and Kemp PJ.** The calcium-sensing receptor beyond extracellular calcium homeostasis: conception, development, adult physiology, and disease. *Annual review of physiology* 74: 271-297, 2012.

37. **Riccardi D, Lee WS, Lee K, Segre GV, Brown EM, and Hebert SC.** Localization of the extracellular Ca(2+)-sensing receptor and PTH/PTHrP receptor in rat kidney. *The American journal of physiology* 271: F951-956, 1996.
38. **Riccardi D, Park J, Lee WS, Gamba G, Brown EM, and Hebert SC.** Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proceedings of the National Academy of Sciences of the United States of America* 92: 131-135, 1995.
39. **Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, and Torok-Storb B.** HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney international* 45: 48-57, 1994.
40. **Satchell SC, Tasman CH, Singh A, Ni L, Geelen J, von Ruhland CJ, O'Hare MJ, Saleem MA, van den Heuvel LP, and Mathieson PW.** Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney international* 69: 1633-1640, 2006.
41. **Tamma G, Lasorsa D, Ranieri M, Mastrofrancesco L, Valenti G, and Svelto M.** Integrin signaling modulates AQP2 trafficking via Arg-Gly-Asp (RGD) motif. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 27: 739-748, 2011.
42. **Tamma G, Robben JH, Trimpert C, Boone M, and Deen PM.** Regulation of AQP2 localization by S256 and S261 phosphorylation and ubiquitination. *American journal of physiology Cell physiology* 300: C636-646, 2011.
43. **Tfelt-Hansen J and Brown EM.** The calcium-sensing receptor in normal physiology and pathophysiology: a review. *Critical reviews in clinical laboratory sciences* 42: 35-70, 2005.
44. **Tyler Miller R.** Control of renal calcium, phosphate, electrolyte, and water excretion by the calcium-sensing receptor. *Best practice & research Clinical endocrinology & metabolism* 27: 345-358, 2013.
45. **Valenti G, Mira A, Mastrofrancesco L, Lasorsa DR, Ranieri M, and Svelto M.** Differential modulation of intracellular Ca2+ responses associated with calcium-sensing receptor activation in renal collecting duct cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 26: 901-912, 2010.
46. **van der Krogt GN, Ogink J, Ponsioen B, and Jalink K.** A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. *PloS one* 3: e1916, 2008.
47. **Vargas-Poussou R, Huang C, Hulin P, Houillier P, Jeunemaitre X, Paillard M, Planelles G, Dechaux M, Miller RT, and Antignac C.** Functional characterization of a calcium-sensing receptor mutation in severe autosomal dominant hypocalcemia with a Bartter-like syndrome. *Journal of the American Society of Nephrology : JASN* 13: 2259-2266, 2002.
48. **Vezzoli G, Terranegra A, Rainone F, Arcidiacono T, Cozzolino M, Aloia A, Dogliotti E, Cusi D, and Soldati L.** Calcium-sensing receptor and calcium kidney stones. *Journal of translational medicine* 9: 201, 2011.
49. **Wagner CA.** The calcium-sensing receptor directly regulates proximal tubular functions. *Kidney international* 84: 228-230, 2013.
50. **Ward DT.** Calcium receptor-mediated intracellular signalling. *Cell calcium* 35: 217-228, 2004.
51. **Wilmer MJ, de Graaf-Hess A, Blom HJ, Dijkman HB, Monnens LA, van den Heuvel LP, and Levtchenko EN.** Elevated oxidized glutathione in cystinotic proximal tubular epithelial cells. *Biochemical and biophysical research communications* 337: 610-614, 2005.
52. **Wilmer MJ, Kluijtmans LA, van der Velden TJ, Willems PH, Scheffer PG, Masereeuw R, Monnens LA, van den Heuvel LP, and Levtchenko EN.** Cysteamine restores glutathione redox status in cultured cystinotic proximal tubular epithelial cells. *Biochimica et biophysica acta* 1812: 643-651, 2011.
53. **Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, Mathieson PW, Monnens LA, van den Heuvel LP, and Levtchenko EN.** Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. *Cell and tissue research* 339: 449-457, 2010.

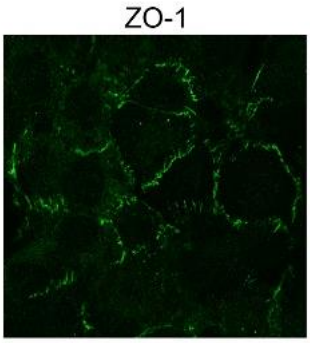
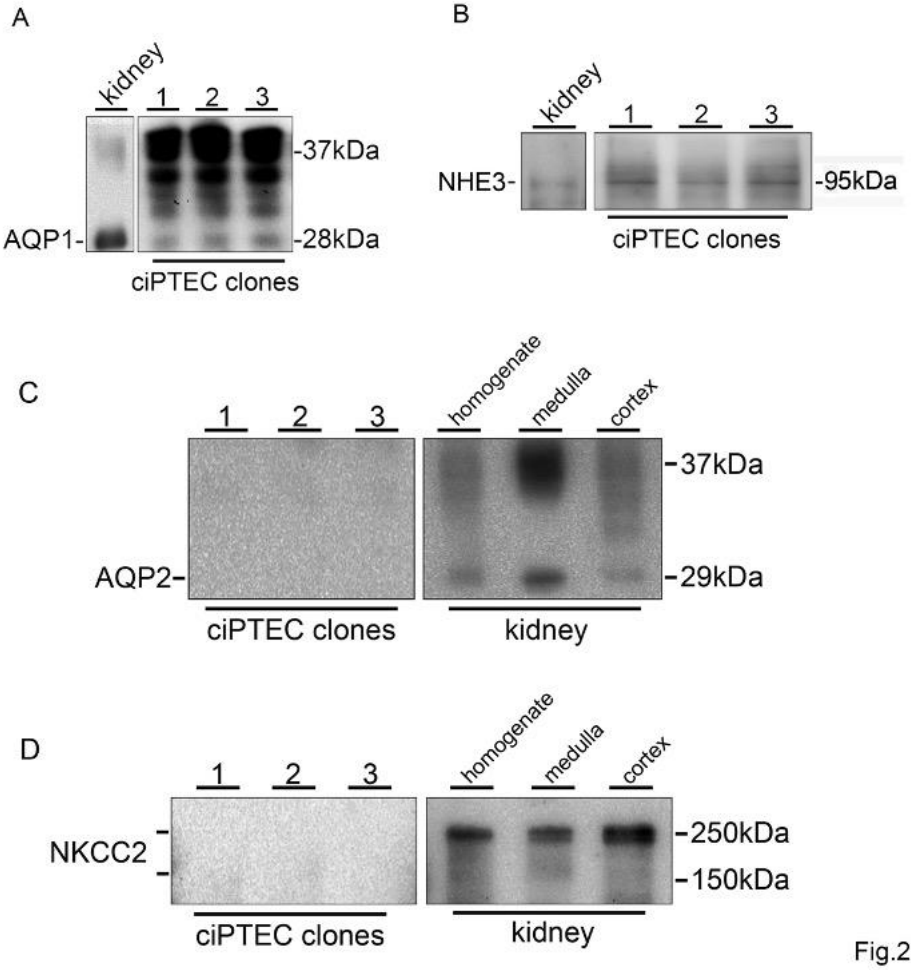

ZO-1

Fig.1


A
kidney
1
2
3
-37kDa
AQP1-
-28kDa
ciPTEC clones
B
kidney
1
2
3
NHE3-
-95kDa
ciPTEC clones
C
1
2
3
homogenate
medulla
cortex
-37kDa
AQP2-
-29kDa
ciPTEC clones
kidney
D
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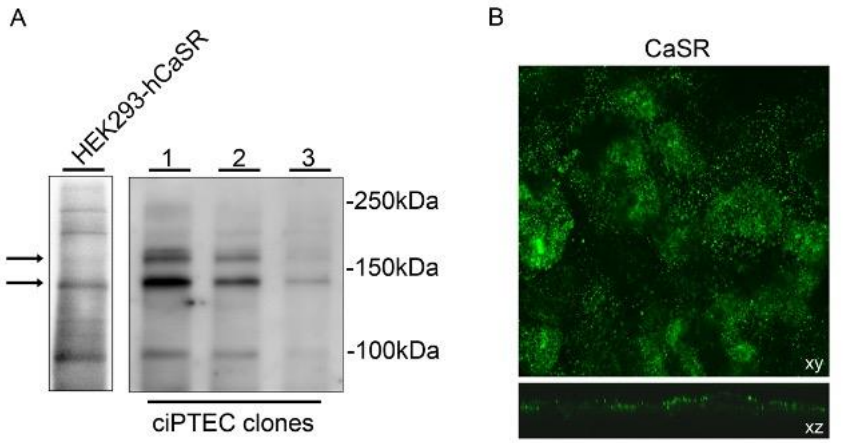
2
3
homogenate
medulla
cortex
NKCC2
-250kDa
-150kDa
ciPTEC clones
kidney

Fig.2



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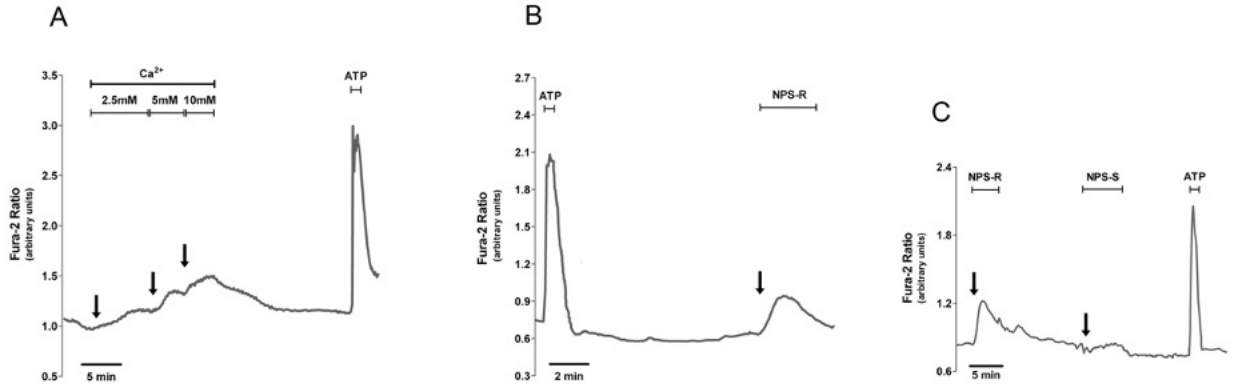

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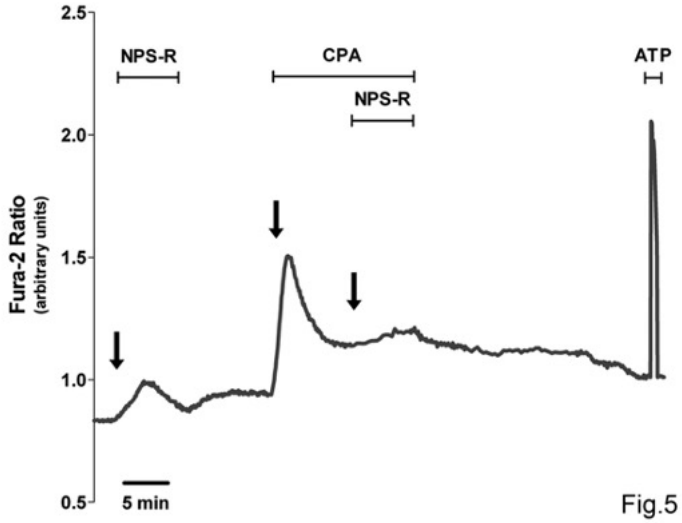

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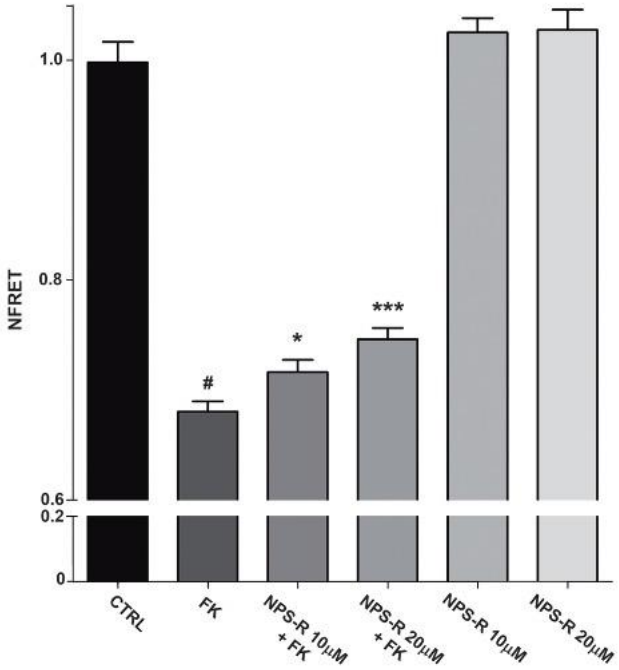
NFRET
1.0
0.8
0.6
0.2
0
#
*
***
CTRL
FK
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NPS-R 20μM + FK
NPS-R 10μM
NPS-R 20μM

Fig.6

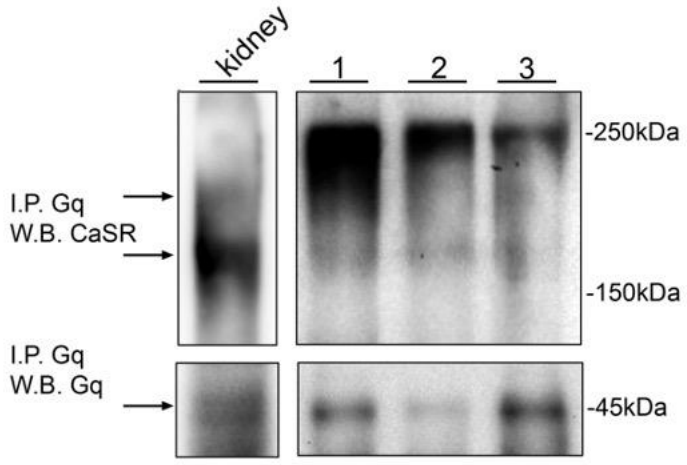
kidney
1
2
3
I.P. Gq
W.B. CaSR
-250kDa
-150kDa
I.P. Gq
W.B. Gq
-45kDa

Fig.7