Conditionally immortalized human proximal tubular epithelial cells isolated from the urine of a healthy subject express functional calcium-sensing receptor

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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 (PLC), causing an accumulation of inositol 1,4,5-trisphosphate (IP3) 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), some 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 discrepancy 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 PTs, 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 (plasma membrane Ca1+-ATPase, 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
The positive allosteric CaSR modulator NPS-R568 resulted in ciPTEC to clinically relevant concentrations of calcium or to nously express CaSR at protein level. Moreover, exposure of characteristics when cultured at the nonpermissive temper-

be maintained for at least 45 passages and present PT mutations by producing cell lines from patients. ciPTEC can able tool for research into the disorder associated with CaSR them (16). These cell lines therefore may represent a valu-

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his possible involvement in prevention of renal stone disease has been postulated. On the basis of this recent evidence, it would be interesting to clarify at the cellular level the molecular mechanism occurring in PT epithelial cells activated by increased luminal calcium concentration leading to enhanced fluid reabsorption in the PT, a process related to activation of CaSR. To this end, a cell line that would be eligible is represented by the condi-

tionally 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 demon-

strate the defect in Na-dependent phosphate reabsorption (52) in cystinosis and altered receptor-mediated endocytosis and the mechanisms of endosomal acidification occurring in them (16). These cell lines therefore may represent a valu-

able 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 nonpermissive temper-

ature of 37°C for 10 days (53).

We provide here the first evidence that ciPTEC endoge-

nously 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 a decrease of FK-

induced 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 PTs.

MATERIALS AND METHODS

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

Antibodies. Monoclonal CaSR antibody recognizing amino acid 15–29 at the extracellular NH2 terminus (17, 47) was from Sigma-Aldrich. To detect AQ2P, we used antibodies against the 20-aminoo-

acid residue segment just NH2 terminal from the polyphosphorylated region of rat AQ2P (CLKGLEEPDTDWEERVRQRQ) (19, 41). Mouse monoclonal NHE3 and rabbit anti-Na+/K+/2Cl− cotrans-

porter (NKCC2) antibodies were from Chemicon (Millipore, Merck, Milan, Italy). Rabbit polyclonal anti-AQP1, mouse anti-zonula oc-

cludens 1 (ZO-1) and mouse anti-Gq antibodies were obtained from Santa Cruz Biotechnologies (TebuBio, Milan, Italy). Secondary mouse antiamphoterin packaging cell line PA 317 (39), containing geneticin (G418) and hygromycin resistance (30, 40). Subconfluent cell layers were transferred to 33°C and selected by using G418 (400 μg/ml) and hygromycin B (25 μg/ml) for 10 days. After being cultured for 2 wk 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% (wt/vol) para-

formaldehyde in HBSS supplemented with 2% (vol/vol) sucrose for 5 min and permeabilized by 10.220.33.5 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from

inhibitors (1 mM PMSF, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM

7.5) in the presence of proteases (1 mM PMSF, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. Cellular debris was removed by centrifugation at 12,000 ×g for 20 min at 4°C. The supernatants were collected and used for immunoblotting studies.

Immunoprecipitation. For immunoprecipitation experiments, ciPTEC were seeded onto 100-mm dishes and grown at 37°C for 10 days. Immunoprecipitation experiments were performed as described (23, 42). Briefly, ciPTEC were lysed with 1% Triton X-100, 150 mM NaCl, and 25 mM Hepes (pH 7.4) in the presence of protease inhibitors (1 mM PMSF, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A). The supernatants were precleared with 50 μl of immobilized proteinc-A and incubated overnight with anti-Gαi antibodies coupled to protein A-Sepharose. As negative control, lysates were incubated with nonspecific rabbit IgG. The immunocomplexes were washed three times,
resuspended in 50 μl 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) for Western blot analysis, blocked in TBS-Tween-20 containing 3% BSA and incubated with primary antibodies overnight. Immunoreactive bands were detected with secondary antibodies conjugated to horseradish peroxidase obtained from Santa Cruz Biotechnologies (Tebu-Bio, Milan, Italy). Membranes were developed using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) with Chemidoc System (Bio-Rad Laboratories, Milan, Italy).

Video-imaging experiments. ciPTEC were grown on 40-mm glass coverslips at 37°C for 10 days and then were loaded with 4 μM fura-2 AM for 15 min at 37°C in DMEM. Ringer’s solution was used to perfuse cells during the experiment containing 120 mM NaCl, 4 mM KCl, 15 mM NaHCO3, 1 mM MgCl2, 15 mM Hepes, 0.5 mM Na3HPO4, 10 mM glucose, 1 mM CaCl2, 0.5 mM Na2HPO4, and 0.4 mM MgSO4, pH 7.4 (modified from Refs. 25 and 26). In fluorescence measurements, the coverslips with dye-loaded cells were mounted in a perfusion chamber (FCS2 Closed Chamber System; BIOPTECHS, Canada). The ratio of fluorescence intensities at 340 and 380 nm was plotted.

Fluorescence resonance energy transfer measurements. For evaluation of cAMP levels in ciPTEC, fluorescence resonance energy transfer (FRET) experiments were performed as described (41). Briefly, ciPTEC were seeded onto 20-mm glass coverslips at 37°C for 10 days and transiently transfected with a plasmid (0.4 μg of DNA/cm2) encoding the H96 probe containing cAMP-binding sequence of Epac1 between cyan fluorescent protein (CFP) and cp173Venus-Venus (gift from Dr. K. Jalink) (46), using Lipofectamine (1 μg/μl) according to the protocol provided by the manufacturer (Life Technologies). Experiments were performed 48 h posttransfection.

After overnight treatment with indomethacin (5 × 10−5 M), a prostaglandin synthesis inhibitor that reduced basal cAMP, cells were left under basal condition or stimulated with FK (10−5 M for 5 min) or alternatively with NPS-R568 (10 μM for 30 min or 20 μM for 15 min) and left unstimulated or stimulated with FK (10−5 M for 5 min).

All treatments were performed in Ringer’s solution described above, containing 1.5 mM CaCl2.

FRET measurements were carried out using MetaMorph software (Molecular Devices, MDS Analytical Technologies). 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 ciPTEC. Because 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. 2, A and B) (29). Cells originating from other segments known to express CaSR that were contaminated were excluded by immunoblotting experiments performed using NKCC2 and AQP2 as markers of the TAL and the collecting duct, respectively. Whereas AQP2 (Fig. 2C) and both forms of NKCC2 at 250 and

Fig. 2. A and B: expression of the proximal tubule-specific proteins. Western blotting of aquaporin 1 (AQP1) (A) and Na+/H+ exchanger 3 (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 and D: expression of AQP2 (C) and Na+/K+/2Cl− cotransporter (NKCC2) (D) markers of the collecting duct and the thick ascending limb of Henle, respectively. Both proteins were not detected in ciPTEC homogenates, confirming the absence of contamination of cells originating from other segments. Western blotting of mice kidney homogenate, medulla, and cortex are shown as positive controls.
160 kDa, corresponding to the mature and the monomeric form (Fig. 2D), were detected in mouse 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. The monomeric form at 130 kDa, the glycosylated monomeric one at 160 kDa, and the dimeric receptor at 250 kDa were detected in ciPTEC clones grown at 37°C for 10 days as well as in HEK293 cells transfected with human CaSR wild-type used as positive control. 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 the 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-2 AM (4 μM) and exposed to raising concentrations of the CaSR physiological agonist, specifically to 2 mM, 5 mM, and 10 mM 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 2 mM [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 10 μM 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 2.5 μM Ca^{2+} and 37.48 ± 1.71% (vs. ATP 100%, n = 25) after stimulation with 10 μM NPS-R568.

To investigate the origin of the calcium release upon specific CaSR activation with NPS-R568, calcium was depleted from the endoplasmic reticulum (ER) using 40 μM cyclopiazonic acid, an inhibitor of the sarco-endoplasmatic reticulum calcium ATPase (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 a CAMP-binding sequence of Epac1 between CFP and Venus. Cells were treated with 10 μM FK, a direct activator of adenylate cyclase, for 5 min or with the positive allosteric CaSR modulator NPS-R568, 10 μM for 30 min or 20 μM for 15 min, or with both NPS-R568 10 μM or 20 μM and FK 10 μM in the last 5 min. CaSR activation with NPS-R signifi-

![Fig. 3. Expression and localization of calcium-sensing receptor (CaSR). A: immunodetection of CaSR in homogenates of ciPTEC clones, after 10 days of maturation at 37°C. Specific anti-CaSR antibodies revealed the monomeric form at 130 kDa, the glycosylated monomeric one at 160 kDa, and the dimeric receptor at 250 kDa compared with CaSR expression in HEK293 lysates, transiently transfected with human CaSR wild-type used as positive control. B: immunofluorescence localization of CaSR in polarized ciPTEC showing its predominant apical plasma membrane localization.](http://ajprenal.physiology.org/attachment/fig3.png)

![Fig. 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 10 μM (B). Cells were also treated with the much less potent stereoisomer NPS-S568 10 μM (C), confirming the specific activation of CaSR elicited by NPS-R568 (28). Fluorescence ratio 340/380 nm was recorded. Each trace is representative of 3–4 different experiments with similar results.](http://ajprenal.physiology.org/attachment/fig4.png)
cantly reduced FK-induced cAMP increase in a dose-depen-
dent manner (Fig. 6). No changes in cAMP levels were
detected in NPS-R568 10 μM- or 20 μM-treated cells with
respect to cells left under basal condition.

CaSR expressed in ciPTEC interacts with Gq. The extracel-
lar calcium-sensing receptor belongs to the C family of the G
protein-coupled receptors and has been shown to couple to Gq
in several tissues (5, 6, 18, 50). To evaluate whether CaSR
expressed in ciPTEC is functionally coupled with Gq, coin-
munoprecipitation studies were performed. ciPTEC lysates
were immunoprecipitated with specific anti-Gq antibodies, and
immunoprecipitates were next probed with anti-CaSR antibod-
ies, revealing positive CaSR bands in all conditions corre-
sponding to the glycosylated monomeric form at
160 kDa and the dimeric receptor at ~250 kDa. These studies indicate that
CaSR couples with Gq as a downstream effector in ciPTEC
(Fig. 7).

DISCUSSION

In this study, we provide the first evidence that ciPTEC
isolated from urine of a healthy volunteer endogenously ex-
press a functional CaSR.

The immortalization of noninvasively collected cells de-
veloped by Wilmer and coworkers (53) has enabled the produc-
tion of human cells maintaining PT characteristics and prolif-
erating for at least 45 passages. For the culture of renal cells
from urine, Wilmer and co-workers have used the immortal-
ization methodology by using SV40T, originally described by
Racusen and colleagues (32). The authors have cultured
ciPTEC clones from the urine of two patients with nephro-
pathic cystinosis, an inherited disorder of PT transport attrib-
utable to lysosomal cysteine accumulation. The detailed char-
acterization of the ciPTEC, including their viability, prolif-
eration 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 col-
lecting cells from the urine of patients (53). Moreover, the
expression pattern and the function of the majority of PT
transporters were similar between cells isolated from urine
compared with those isolated from kidney tissue and immor-
talized 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 reabsorp-
tion, demonstrating that CLC-5 mutations have multiple

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

Fig. 6. Evaluation of cAMP levels in proximal tubular epithelial cells by
fluorescence resonance energy transfer (FRET) analysis. Histograms compare
changes of nonoverlapping FRET (NFRET) ratio under different experimental
conditions. Forskolin (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 10 μM and 20 μM (*P < 0.01 and ***P < 0.0001, respec-
tively). NPS-R568 did not alter cAMP levels under basal conditions.

Fig. 7. Coimmunoprecipitation of Gq and CaSR proteins. ciPTEC clone
homogenates were subjected to immunoprecipitation (I.P.) with specific anti-
body against Gq. Immunoprecipitates were probed with anti-CaSR antibody.
Positive CaSR bands corresponding to the glycosylated monomeric form at
160 kDa and the dimeric receptor at ~250 kDa were detected. CaSR and Gq
expression in rat kidney homogenate is shown as positive control. W.B.,
Western blot.
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 noninvasive 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 exacerbated signalling (33).

In the aim of using ciPTEC obtained by immortalizing and subcloning cells exfoliated in the urine of a 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 ZO-1 of the AQP1 and NHE3 proteins, proving their epithelial and PT origin, respectively. The specific derivation of this clonal cell line from PT was demonstrated by the absence of other tubular segment protein 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 with CaSR expressed in ciPTEC. The reported data obtained by coimmunoprecipitation studies identified Gq as one of the possible downstream effectors of CaSR in ciPTEC.

It is known that CaSR can couple to several G proteins, such as 1) Gq/11, which stimulates PLC, thereby producing diacylglycerol and IP3, causing releases of calcium from intracellular stores; 2) Gi, resulting in adenylate cyclase inhibition; and 3) G12/13, 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 PT causes an increase in intracellular calcium attributable to CaSR coupling to Gq, 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.D.M. and M.R. performed experiments; A.D.M., G.T., M.R., M.S., and G.V. interpreted results of experiments; A.D.M. and G.V. drafted manuscript.

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