Expression and Function of the Calcium-Sensing Receptor in Juxtaglomerular Cells

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Abstract—Calcium-sensing receptors sense and translate micromolar changes of extracellular calcium into changes in intracellular calcium. Renin, a component of the renin-angiotensin system, is synthesized by, stored in, and released from the juxtaglomerular cells through a cAMP-dependent pathway. Increased intracellular calcium inhibits the adenylyl cyclase isoform type V, cAMP formation, and renin release from juxtaglomerular cells. We hypothesized that calcium-sensing receptors are expressed in juxtaglomerular cells and mediate changes in intracellular calcium and renin release. To test this we used primary cultures of isolated mouse juxtaglomerular cells in which we ran RT-PCR, Western blots, and immunofluorescence. RT-PCR showed a positive band at the expected 151 bp consistent with calcium-sensing receptor. Western blots showed a 130- to 150-kDa band confirming the calcium-sensing receptor in juxtaglomerular cells. Immunofluorescence and confocal microscopy using 2 different antibodies against the calcium-sensing receptor in juxtaglomerular cells showed positive fluorescence in the juxtaglomerular cells, which also had positive labeling for renin. To test whether calcium-sensing receptors regulate renin release, juxtaglomerular cells were incubated with a calcium-sensing receptor agonist, the calcimimetic cinacalcet-HCl, at concentrations of 50 and 1000 nmol/L in 0.25 mmol/L of calcium medium. Cinacalcet-HCl decreased juxtaglomerular cell cAMP formation to 47.3±6.8% and 44.2±9.7% of basal, respectively (P<0.001), and decreased renin release from 541.9±86.2 to 364.6±64.1 (P<0.05) and 279.6±56.9 (P<0.005) ng of angiotensin I per milliliter per hour per milligram of protein, respectively. We conclude that juxtaglomerular cells express the calcium-sensing receptor and that their activation leads to inhibition of adenylyl cyclase-V activity, decreasing cAMP formation and suppressing renin release. (Hypertension. 2007; 50:737-743.)

Key Words: renin ▪ calcium-sensing receptor ▪ cAMP ▪ calcium ▪ juxtaglomerular cells
medullary collecting duct cells. However, the presence of CaSRs on JG cells has not been described.

In the present study we hypothesized that the decrease in renin secretion by JG cells in response to increased extracellular calcium is mediated by CaSRs on the JG cells. Changes in extracellular calcium are translated through the CaSRs into an intracellular signal. Stimulation of the CaSRs should result in inhibition of AC-V, decreased cAMP formation, and decreased renin release. We present evidence that CaSRs are expressed in JG cells and, further, that their activation by a calcimimetic leads to a decrease in cAMP formation and the suppression of renin release.

Methods

Isolation of Mouse JG Cells

The source for our JG cells were 8- to 10-week-old C57/BL6 mice obtained from Jackson Laboratories (Bar Harbor, Maine). These procedures were reviewed by our Institutional Animal Care and Use Committee and adhere to the guiding principles in the care and use of experimental animals and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Henry Ford Hospital’s animal facility is Associated for Assessment and Accreditation of Laboratory Animal Care International-approved. JG cells were isolated using a technique derived from the methods of della Bruna et al,15 with numerous modifications as we have described previously in detail.9,10 Once isolated, JG cells were incubated for 48 hours at 70% to 80% confluence. After that, the incubation medium was replaced with serum-free medium to carry out experimental protocols (outlined below).

CaSR Expression in JG Cells

RT-PCR for CaSR

RT-PCR for CaSR was run using isolated mouse JG cells. A suspension of the mouse renal cortex was used as a positive control.16 A “no-template” control was used as a negative control. Isolated JG cells were washed with PBS and resuspended in 1 mL of Tri reagent. Likewise, the mouse kidney cortex was snap frozen in liquid nitrogen, followed by homogenization in 1 mL of Tri reagent. One microgram of extracted total RNA was reverse transcribed at 37°C using Omniscript (Qiagen), and 2 μL of the mixture were used for subsequent PCR. The following primers, adapted from Yao et al (EMBL/GenBank accession No. AF110178), were used to detect the CaSR: sense 5’ aacacatcagccacgca 3’ and antisense 5’ tagtgtatcactcctgga 3’. PCR was performed under the following conditions: 94°C for 2 minutes; 40 cycles of 95°C for 30 seconds, 56°C for 40 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The reaction products were then held at 4°C. PCR products were run on a 2% agarose gel in 1× TBE (90 mM/50 mM Tris/64.6 mM/100 mM EDTA (pH 8.3)). The CaSR gives a PCR product at 151 bp.

Western Blot for CaSR

Isolated JG cells were resuspended in lysis buffer (50 mMol/L Tris (pH 6.8)/5% [vol/vol] glycerol/2% SDS) containing a protease inhibitor mixture and incubated for 10 minutes on ice (as described previously).15,16 Likewise, dissected rat parathyroid was snap frozen in liquid nitrogen and then homogenized with the lysis buffer referred to above containing the protease inhibitor mixture. Solubilized JG cell and parathyroid proteins (2 and 5 μg each) were heated to 95°C for 5 minutes, and the cell lysate was subjected to polyacrylamide gel electrophoresis under reducing conditions. Proteins were electrophoretically transferred to a PVDF membrane overnight at 4°C. The membranes were blocked for 1 hour at room temperature and incubated with a specific antibody against CaSR (Affinity Bioreagents) diluted 1:50 overnight at 4°C. The presence of CaSR was detected using a horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody at a dilution of 1:5000. The bands were identified by chemiluminescence and exposed to x-ray film. These experiments were repeated 3 times.

Immunolabeling of CaSR in Nonpermeabilized JG Cells

To demonstrate the presence of CaSR in the JG cells, after JG cells were incubated for 48 hours to collagen IV–coated coverslips (Trevigen Inc), the culture medium was removed and replaced with TBS Tween-20 (TBST) 5% BSA with the CaSR antibody (Affinity Bioreagents) at a 1:50 dilution and incubated overnight at 4°C. The cells were fixed for 30 minutes with freshly prepared 4% paraformaldehyde diluted in PBS then washed with TBST 3 times for 5 minutes each, then incubated with a secondary goat anti-mouse antibody labeled with Alexa Fluor 568 red-orange fluorescent dye (Invitrogen), diluted 1:100 in 5% BSA in TBST for 1 hour. After incubation with the secondary antibody, cells were washed and the coverslips mounted on slides with Fluoromount (Southern Biotech Associates). The following controls were conducted to test the binding specificity: primary and secondary antibodies were replaced by TBST 5% BSA, or only the primary antibody was replaced by TBST 5% BSA. The preparations were examined by confocal microscopy (Visitech Confocal System). Samples were excited at 568 nm and emission measured at >590 nm. These experiments were repeated 5 times.

Immunolabeling of Renin in JG Cells

For the demonstration of renin, similar to our previous reports,9,10 JG cells grown on coverslips were fixed for 30 minutes with freshly prepared 4% paraformaldehyde diluted in PBS and then washed with TBST 3 times for 5 minutes each. The fixed cells were permeabilized with PBS containing 0.1% Triton X-100 for 20 minutes and then washed. Nonspecific binding sites were blocked with TBST and 5% BSA for 30 minutes and then washed. The cells were incubated for 2 hour with a primary antibody against mouse renin18 (Swant) diluted 1:50 in TBST containing 5% BSA. Cells were then washed and incubated with a 488 fluorescent dye goat anti-mouse (Alexa Fluor, Invitrogen) secondary antibody diluted 1:100 in TBST with 5% BSA for 1 hour. After incubation with secondary antibody, cells were washed, and the coverslips were mounted in slides with Fluoromount (Southern Biotech Associates, Inc). The following controls were made to test the specificity of the binding: primary and secondary antibodies were replaced by TBST with 5% BSA, or only primary antibody was omitted and replaced by TBST with 5% BSA. The preparations were examined by confocal microscope (Visitech Confocal System) excited at 488 nm, and then emission was measured at >500 nm.

Colocalization of CaSR and Renin

To ensure that our identification of CaSR was in JG cells and not the result of contamination, we counted the number of cells (as a percentage of total cells) that labeled for both renin and for CaSR, obtaining counts from 6 different fields. Counts were done using Intervision 2D analysis software. CaSR-positive and renin-positive cells are presented as a percentage of the total cells per field.

Immunolabeling of CaSR and Renin

To further confirm that CaSR is in JG cells, we did colocalization studies with the same protocol described for CaSR and renin immunolabeling (see above) with the following changes. We used a different anti-CaSR primary antibody raised in rabbits rather than mice (MBL International) at 1:50 in TBST 5% BSA, and the secondary antibody was goat anti-rabbit labeled with Alexa Fluor 568 red-orange fluorescent dye (Alexa Fluor, Invitrogen) diluted 1:100 in TBST 5% BSA. Cells were first incubated with the CaSR antibody and then incubated with the renin antibody.16 The immunolabeling protocol is identical to that we have published previously.15,16 Mounted samples were excited at 488 nm and emission measured at >500 nm to obtain images of the renin antibody and at 568 nm and emission measured at >590 nm for the CaSR antibody.
CaSR Stimulation With Cinacalcet-HCl
After incubating isolated JG cells for 48 hours, the culture medium was switched to serum-free Minimum Essential Medium (Gibco-Invitrogen) plus calcium added at a 0.25-mmol/L concentration, containing the following: (1) 100 μmol/L of 3-isobutyl-L-methyl-xanthine (control), (2) 100 μmol/L of 3-isobutyl-L-methyl-xanthine plus 50 mmol/L of cinacalcet-HCl, or (3) 100 μmol/L of 3-isobutyl-L-methyl-xanthine plus 1000 mmol/L of cinacalcet-HCl. After 2 hours, medium was sampled for renin determination, and then cells were harvested for determination of intracellular cAMP (n = 8).

Renin Release From Isolated Mouse JG Cells
JG cells were incubated with serum-free medium containing 100 μmol/L of 3-isobutyl-L-methyl-xanthine (Sigma) for 2 hours with cinacalcet or its vehicle. The medium was then drawn off and centrifuged and the supernatant recovered for assay of renin concentration (angiotensin I generated per milliliter of sample per hour of incubation).

The cAMP content was determined from the harvested cells using a colorimetric immunoassay kit (Pierce Biotechnology Inc). Values for sheep angiotensinogen and assayed using a Gamma Coat radioimmunoassay kit (Pierce Biotechnology Inc). Values for renin concentration were corrected for JG cell total protein. The protein concentration in JG cellular lysates was determined using a Coomassie plus Protein Assay Reagent kit (Pierce Biotechnology Inc).

Statistical Analysis
Analysis of the changes in both JG cell renin release and cAMP concentration were measured by ANOVA with a Bonferroni posthoc test. We considered an adjusted P<0.05 to be significant.

Results

CaSR Expression in JG Cells
RT-PCR for CaSR
We first assessed whether CaSR expression was present in JG cells by RT-PCR. Figure 1 show that the RT-PCR performed on 1 μg of total RNA gave a product at the expected size of 151 bp in both isolated JG cells and in the positive control of mouse cortex. The no-template negative control showed no amplification. These results suggest that there is expression of CaSR in JG cells. The PCR product was verified to be the calcium-sensing receptor after sequencing by the University of Michigan Core Sequencing facility. Using a basic local alignment search tool, sequences derived from both the forward and reverse primers gave only 1 transcript, that of the Mus musculus calcium sensing receptor (NM 013803.1/AF110178).

Western Blot for CaSR
Western blot performed using the CaSR antibody identified a band at 130 to 150 kDa that corresponds with the expected size of CaSR (Figure 2). The antibody also identified CaSR in the positive control of the dissected parathyroid gland. The broader band for CaSR seen in JG cells suggests a doublet, possibly because of different glycosylation states of the CaSR in our preparation. These results further support the presence of CaSR in JG cells.

Immunolabeling of CaSR in Nonpermeabilized JG Cells
Immunofluorescence and confocal microscopy performed in isolated JG cells incubated with the CaSR antibody are shown in Figure 3. The images show a homogeneous pattern of labeling throughout the JG cell and not a discrete localization on the cell membrane, as expected. The cells were not permeabilized, and they were incubated with the primary antibody before being fixed at 4°C. The images showed intense fluorescence throughout the JG cells, further supporting expression of CaSR in JG cells.

Colocalization of CaSR and Renin
We found that cells with CaSR-positive immunolabeling accounted for 94±9% of the total counted. This compared with 83±4% for renin-positive cells. The greater staining with the CaSR antibody is expected, because non-JG contamination from the renal cortex is likely to express the CaSR. However, these data also suggest that the majority of the cells that stain for CaSR are also renin-containing JG cells.

Coimmunolabeling of CaSR and Renin
In an additional experiment to insure that our identification of CaSR was in JG cells and not the result of non-JG cell contamination, coimmunolabeling of both CaSR and renin by immunofluorescence and confocal microscopy with antibodies against CaSR and against renin are presented in Figure 4. Panel A shows the localization of CaSR, which seems to be dispersed throughout the cell, similar to what was seen using the other CaSR antibody (Figure 3). Panel B represents a transmitted light image of the same JG cell in primary culture. Panel C shows the localization of renin in the same cell, in green. The distribution of renin appears in granule-like foci throughout the cytoplasm, similar to what we have reported previously in primary cultures of isolated JG cells. Thus, not only do we find CaSRs in our preparation using 2 different antibodies, but we clearly show that they are expressed in the renin-containing JG cells.
CaSR Stimulation With Cinacalcet-HCl

We stimulated CaSRs on JG cells using CaSR agonist cinacalcet-HCl in moderately low calcium medium and measured cAMP and renin. JG cells were incubated with cinacalcet-HCl at concentrations of either 50 or 1000 nmol/L for 2 hours. Cinacalcet-HCl decreased cAMP to 47.3 ± 6.8% at 50 nmol/L (P < 0.001) and to 44.2 ± 9.7% with 1000 nmol/L cinacalcet-HCl (P < 0.001; Figure 5A). These changes with cinacalcet-HCl were not different from each other. Renin release (Figure 5B) was reduced from 541.9 ± 86.2 to 364.6 ± 64.1 ng of angiotensin I per milliliter per hour per milligram of protein (P < 0.05) with 50 nmol/L of cinacalcet-HCl and to 279.6 ± 56.9 ng of angiotensin I per milliliter per hour per milligram of protein (P < 0.005) with 1000 nmol/L of cinacalcet-HCl. These changes with cinacalcet-HCl were not different from each other. Thus, activation of CaSR with the agonist leads to decreased cAMP formation and suppressed renin release from JG cells.

Discussion

We show for the first time the presence of calcium-sensing receptors in JG cells, as well as their participation in regulating renin release by decreasing intracellular cAMP formation. These observations include evidence by RT-PCR, Western blots using antibodies targeted to the CaSR, and confocal microscopy and immunolabeling of the CaSR in our preparation of the primary culture of isolated JG cells that we also show to contain renin. We also show the direct inhibition of both renin release and its second messenger cAMP when we stimulate the CaSR using a selective agonist. Together these results using a variety of techniques all support our hypothesis that CaSRs are expressed in JG cells and mediate changes in renin release.

There are 2 secretory cell types in which there is a paradoxical inverse relationship between secretion and intracellular calcium: the parathyroid and the JG cell. The parathyroid gland secretes parathyroid hormone in response to reduced extracellular calcium or suppresses secretion when extracellular calcium is increased. These signals are translated into the intracellular mechanisms controlling secretion through CaSR, which can sense micromolar changes in calcium.21 We hypothesized that these receptors might work in a similar fashion on JG cells.
The CaSR is a G protein–coupled receptor with a large amino-terminal extracellular domain, a transmembrane domain with 7 helices and a cytoplasmic carboxyl terminus (intracellular domain).\textsuperscript{22} N-linked glycosylation sites found in the CaSR are important for its expression on the cell surface, where the receptor resides as a disulfide-linked dimer.\textsuperscript{13} Activation of the CaSR leads to the activation of phospholipases, generation of inositol triphosphate and diacylglycerol, changes in protein phosphorylation, activation of ion channels, and increases in the intracellular calcium concentration.\textsuperscript{23} The CaSR may also inhibit adenylyl cyclase through Gi.\textsuperscript{22} In the kidney, the CaSR has been shown to regulate the movement of water and other electrolytes.\textsuperscript{14} CaSRs are involved in the regulation of renal calcium excretion and to influence the transepithelial potential difference.\textsuperscript{22,24} RT-PCR showed an RT-PCR product at the JG cells. RT-PCR in our JG cells, using renal cortex as a positive control,\textsuperscript{22,24} showed an RT-PCR product at the expected size for the CaSR (151 bp). Although this is a positive result, our preparation has 10% to 15% of non-JG contamination,\textsuperscript{9} which could result in a false-positive, so additional verification was run.

Our results demonstrate that CaSRs are also expressed in JG cells. RT-PCR in our JG cells, using renal cortex as a positive control,\textsuperscript{22,24} showed an RT-PCR product at the expected size for the CaSR.\textsuperscript{(151 bp)} Although this is a positive result, our preparation has 10% to 15% of non-JG contamination,\textsuperscript{9} which could result in a false-positive, so additional verification was run.

Western blots confirmed the presence of the characteristic band reported for the antibody that we used at 130 to 150 kDa.\textsuperscript{17} We used the parathyroid gland as a positive control, because this is where CaSR was initially described.\textsuperscript{22} The native CaSR can exist in 3 forms: (1) a 120-kDa band, which represents the nonglycosylated species; (2) a 140-kDa band, which represents the immature glycosylated receptor; and (3) a 160-kDa band, which is the mature, fully glycosylated receptor.\textsuperscript{25} We observed a wider band in the Western blot of the JG cells compared with parathyroid (Figure 2), which may be because of multiple forms existing in our preparation.

Immunofluorescence using an antibody specific to the CaSR also confirmed the presence of CaSR in the JG cells (Figure 3). We expected to find immunolabeling localized to the JG cell membrane but observed it also diffusely distributed throughout the cytoplasm (Figure 3). Although these cells were not permeabilized, and the incubation with the antibody was performed at 4°C before fixing them with 4% paraformaldehyde, it is possible that the CaSR on the JG cells internalized the antibody over the incubation period. As reported previously,\textsuperscript{9,10} these cells from our preparation also contain renin. To insure that we were actually studying renin-containing JG cells, we used a second antibody against CaSR and found colocalization of CaSR with renin.

Based on the parathyroid as a model, we proposed that the existence of CaSR on JG cells could explain the mechanism by which these cells sense and translate changes in the extracellular or interstitial calcium concentration into changes in intracellular calcium that can influence the activity of AC-V and subsequently regulate the release of renin. We used the calcimimetic cinacalcet-HCl to test our hypothesis, because this is a ligand that mimics or enhances the effects of extracellular calcium at the CaSR. We show that activation of the CaSR in JG cells leads to a significant decrease in intracellular cAMP formation and also renin release as hypothesized.

There are 2 mechanistically distinct types of calcimimetics. Type I calcimimetics are agonists and include inorganic and organic polycations, whereas type II calcimimetics are allosteric activators that interact with the membrane-spanning segments of the CaSR and enhance signal transduction, presumably by inducing conformational changes in the receptor so as to reduce the threshold for CaSR activation by the endogenous calcium. Cinacalcet-HCl is a type II agonist.\textsuperscript{19} We selected 2 different concentrations of the agonist to stimulate CaSR in the isolated JG cell culture from the literature. The higher dose of cinacalcet-HCl (1000 nM) chosen was obtained from Kawata et al,\textsuperscript{26} in which its inhibitory effect on parathyroid hormone secretion was observed in primary cultured parathyroid cells obtained from patients. The lower concentration of 50 nM selected was based on the IC\textsubscript{50} of the cinacalcet.\textsuperscript{19,27} The calcium concentration in the incubation medium was based on the normal extracellular free-ionized calcium concentration of 1.2 mmol/L, which is also the EC\textsubscript{50} for the CaSR.\textsuperscript{20} We expected that calcimimetics, such as cinacalcet-HCl, would shift the concentration-response curves for extracellular calcium and increase its sensitivity to activation by extracellular calcium.\textsuperscript{19} Both concentrations of the agonist used gave similar results, suggesting that the JG cells are very sensitive to the signal, because 50 nmol/L of calcium is enough to activate CaSR.
The observation that the extracellular calcium concentration had a direct but paradoxically inverse influence on renin secretion was first made in 1977 by Fray et al. using different perfusate calcium concentrations in an isolated perfused kidney. Numerous additional studies in the following decade by Fray and others (as reviewed by Fray et al.) found a parabolic increase in either basal or stimulated renin release as extracellular calcium declined from high (7 to 8 mmol/L) to low (0) concentrations. Importantly, the renin released at normal calcium concentrations (~2.5 mmol/L) fell near the middle of this curve, and the value 1.25 mmol/L reported for ionized free calcium in the renal cortical interstitium falls on a steeper part of the curve, all suggesting that very small, micromolar changes in the extracellular calcium can elicit a significant effect on the rate of renin secretion at or near normal calcium levels. This is important in that the CaSR is responsive to micromolar changes in extracellular calcium, suggesting that such small changes in the extracellular environment can realistically influence the intracellular calcium signaling that we propose in the JG cell within normal physiological parameters.

Previously we found that the calcium-inhibitable AC-V but not type VI is expressed in the JG cells. It showed a similar distribution within the JG cell cytoplasm as renin, colocalizing on the renin granules. Decreasing intracellular calcium with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate-AM resulted in increased JG cell cAMP formation and renin release, and these responses could be blocked by a selective AC-V inhibitor. We have not shown that activation of the JG cell CaSR actually results in an increase in intracellular calcium. As discussed earlier, the CaSR affects multiple intracellular pathways, including decreasing the intracellular calcium concentration. These results are consistent with our previous observations so that an increase in intracellular calcium could inhibit AC-V activity, decreasing in intracellular cAMP formation and renin release. However, it remains to be shown whether CaSR activation on JG cells is mediated by changing intracellular calcium or results from multiple postreceptor responses. Regardless, it is clear that the JG cells do express CaSR and that they do mediate cAMP formation in and renin release from the JG cells.

This study proposed that the JG cells, much like the parathyroid glands, have a CaSR, which may participate in the paradoxical relationship between intracellular calcium and renin secretion. Our novel results confirm that CaSRs are expressed in JG cells and mediate changes in adenylyl cyclase activity, cAMP formation, and renin release.

**Perspectives**

It has been understood since the seminal work of Fray that the intracellular calcium concentration in the JG cell served to regulate the secretion of renin. However, despite numerous studies, the mechanism(s) by which this occurred has remained illusive. The discovery of the calcium-inhibitable AC-V in the JG cell, now followed by our novel report of CaSR expressed by the JG cell, presents some provocative new answers as to how this unique regulation of renin secretion occurs. But what parameter would target these JG cell CaSRs? It is unlikely due to calcium in the circulation, which is closely regulated. However, it has been reported that chronic increases in distal NaCl delivery increase the renal cortical interstitial calcium. Such a response would provide a pathway for calcium signaling of the JG cell CaSR, consistent with the well-established effects of NaCl on renin secretion. Although seemingly logical, this provocative possible signaling cascade remains to be tested.

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**Disclosures**

None.

**References**

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