Juxtaglomerular cell CaSR stimulation decreases renin release via activation of the PLC/IP₃ pathway and the ryanodine receptor

M. Cecilia Ortiz-Capisano,1 Mahendranath Reddy,1 Mariela Mendez,1 Jeffrey L. Garvin,1,2 and William H. Beierwaltes1,2

1Hypertension and Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, Michigan; and 2Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan

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The calcium-sensing receptor (CaSR) is a G-coupled protein expressed in renal juxtaglomerular (JG) cells. Its activation stimulates calcium-mediated decreases in cAMP content and inhibits renin release. The postreceptor pathway for the CaSR in JG cells is unknown. In parathyroids, CaSR acts through Gq and/or G₁i. Activation of Gq stimulates phospholipase C (PLC), and inositol 1,4,5-trisphosphate (IP₃), releasing calcium from intracellular stores. G₁i stimulation inhibits cAMP formation. In afferent arterioles, the ryanodine receptor (RyR) enhances release of stored calcium. We hypothesized JG cell CaSR activation inhibits renin via the PLC/IP₃ and also RyR activation, increasing intracellular calcium, suppressing cAMP formation, and inhibiting renin release. Renin release from primary cultures of isolated mouse JG cells (n = 10) was measured. The CaSR agonist cinacalcet decreased renin release by 56 ± 7% of control (P < 0.001), while the PLC inhibitor U73122 reversed cinacalcet inhibition of renin (104 ± 11% of control). The IP₃ inhibitor 2-APB also reversed inhibition of renin from 56 ± 6 to 104 ± 11% of control (P < 0.001). JG cells were positively labeled for RyR, and blocking RyR reversed CaSR-mediated inhibition of renin from 61 ± 8 to 118 ± 22% of control (P < 0.01). Combining inhibition of IP₃ and RyR was not additive. G₁i inhibition with pertussis toxin plus cinacalcet did reverse renin inhibition (65 ± 12 to 41 ± 8% of control, P < 0.001). We conclude stimulating JG cell CaSR activates Gq, initiating the PLC/IP₃ pathway, activating RyR, increasing intracellular calcium, and resulting in calcium-mediated renin inhibition.

The calcium-sensing receptor (CaSR) is a G protein-coupled 7-transmembrane receptor (33) found in the parathyroid gland, blood vessels, and kidney (18). In the parathyroid gland where CaSR was first described, high extracellular calcium results in decreased parathyroid hormone secretion (21). This is mediated by calcium activation of the CaSR, stimulating the G proteins Gq and/or G₁i. In the parathyroid, activation of Gq stimulates phospholipase C (PLC), producing diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), the latter of which releases calcium from intracellular stores bound in the endoplasmic reticulum (ER) (48). G₁i stimulation leads to the inhibition of cAMP formation (16, 48). Either pathway results in suppressing parathyroid hormone release.

Address for reprint requests and other correspondence: W. H. Beierwaltes, Dept. of Internal Medicine, Hypertension and Vascular Research Div., Henry Ford Hospital, 7121 E&R Bldg., 2799 W. Grand Blvd., Detroit, MI 48202 (e-mail: wbeierw1@hfhs.org).
cAMP formation (11), we also tested the possible involvement of G_{i} in the CaSR-mediated inhibition of renin release.

**MATERIALS AND METHODS**

**Primary Culture of Isolated JG Cells**

*Isolation of mouse JG cells.* This study conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All of our protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Henry Ford Health System.

We used primary cultures of mouse isolated juxtaglomerular (JG) cells, with a protocol modified in our laboratory (43–46) to improve the harvest, purity, and stability of the primary culture (39). The JG cells were incubated at 37°C in a humidified atmosphere containing 5% CO_{2} in air. After 48 h of incubation, the culture medium was removed, and 250 μL of fresh, prewarmed serum-free culture medium containing 1.2 mM calcium (or alternative ionized free calcium concentrations as described below) was added, along with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, Sigma, St Louis, MO) dissolved in DMSO (Sigma, St Louis, MO). Experiments were performed in this medium. JG cells were incubated for 2 h, after which the supernatant was collected, centrifuged to remove any cellular debris, and assayed for the activity of renin released into the medium (see below), and in *protocol 1*, JG cells were harvested for cAMP measurements (see below). All protocols utilized paired experiments run on JG cells from the same harvest in a given 24-well culture plate.

We are only presenting cAMP data in our first protocol, as in our previous work (43–46) we have repeatedly shown the consistency of renin release correlating with cAMP content in our isolated JG cell preparation. Since this is well established, and since our end point is renin release, *protocols 2–5* only report renin. Additionally, the CaSR-mediated changes in intracellular calcium, while well established, are not measured directly. Previously, our laboratories have made extensive efforts to directly study the changes in intracellular calcium in JG cells using fluorescent dyes. However, we discovered that in our isolated JG cells or in microdissected afferent arterioles, the dyes are quickly compartmentalized in the cytoplasm, making such measurements impossible. We used several intracellular calcium indicators, including fura-2, calcium green, and fluo-4 (Invitrogen, Molecular Probes, Eugene, OR) (54) all in the AM form, which entered the JG cells but were quickly taken up into granules, not allowing the esterase to cleave the AM group to bind to the intracellular calcium (unpublished observations). This is in contrast to the studies performed in the adjacent afferent vascular smooth muscle cells that work well with such calcium dyes (26). We suggest that any cell responding to the dyes was vascular smooth muscle and not a JG cell. Thus we do not (cannot) measure intracellular calcium directly in this preparation.

We placed our primary cultures of JG cells on poly-D-lysine-coated cover slips for 48 h. The medium was then removed and the cells fixed for 30 min with freshly prepared 4% paraformaldehyde diluted in PBS, then washed with Tris-buffered saline-Tween (TBST) three times for 5 min each. The fixed cells were permeabilized with 0.2% Triton X-100 for 10 min, then washed. Nonspecific binding was blocked with 5% BSA for 30 min. The cells were incubated for 1 h with a RyR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (14) diluted 1:25 in 5% BSA. Cells were then washed, and incubated with a goat anti-rabbit antibody labeled with Alexa Fluor 568 fluorescent dye (Alexa Fluor, Invitrogen) diluted 1:100 in 5% BSA. After incubation with the secondary antibody, cells were again washed and then incubated for 1 h with a 1:25 dilution of an antibody against renin protein (sheep anti-mouse FITC-labeled, Innovative Research). Cells were again washed and the coverslips were mounted on slides with Fluoromount (Southern Biotech Associates). As a negative control we repeated the same procedure but omitted the primary antibody (against RyR) and incubated with only the secondary antibody, and no labeling was seen. The preparations were examined by confocal microscopy (Visitech Confocal System) and excited at 488 nm; emission was measured at >500 nm to obtain images of the renin antibody and at 568 nm, and emission was measured at >590 nm for images of the RyR antibody. This protocol was repeated four times with different preparations, and each time images of at least 10 cells were taken. Captured images were uniformly enhanced for intensity and colored to discriminate renin (green) from RyR (red) using Photoshop Elements 4.0 (Adobe Systems, San Jose, CA), but images were not altered.

**RyR in the CaSR-Mediated Inhibition of Renin Release**

*RyR expression in JG cells: communolabeling of RyR and renin in JG cells (39).* We placed our primary cultures of JG cells on poly-D-lysine-coated cover slips for 48 h. The medium was then removed and the cells fixed for 30 min with freshly prepared 4% paraformaldehyde diluted in PBS, then washed with Tris-buffered saline-Tween (TBST) three times for 5 min each. The fixed cells were permeabilized with 0.2% Triton X-100 for 10 min, then washed. Nonspecific binding was blocked with 5% BSA for 30 min. The cells were incubated for 1 h with a RyR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (14) diluted 1:25 in 5% BSA. Cells were then washed, and incubated with a goat anti-rabbit antibody labeled with Alexa Fluor 568 fluorescent dye (Alexa Fluor, Invitrogen) diluted 1:100 in 5% BSA. After incubation with the secondary antibody, cells were again washed, and then incubated for 1 h with a 1:25 dilution of an antibody against renin protein (sheep anti-mouse FITC-labeled, Innovative Research). Cells were again washed and the coverslips were mounted on slides with Fluoromount (Southern Biotech Associates). As a negative control we repeated the same procedure but omitted the primary antibody (against RyR) and incubated with only the secondary antibody, and no labeling was seen. The preparations were examined by confocal microscopy (Visitech Confocal System) and excited at 488 nm; emission was measured at >500 nm to obtain images of the renin antibody and at 568 nm, and emission was measured at >590 nm for images of the RyR antibody. This protocol was repeated four times with different preparations, and each time images of at least 10 cells were taken. Captured images were uniformly enhanced for intensity and colored to discriminate renin (green) from RyR (red) using Photoshop Elements 4.0 (Adobe Systems, San Jose, CA), but images were not altered.

**IP_{3} inhibition with 2-APB (n = 10).** To study if renin inhibition resulting from CaSR activation is dependent upon the PLC/IP_{3} pathway, we incorporated the IP_{3} inhibitor 2-APB (8, 49) (EMD Chemicals, Philadelphia, PA) into our protocol, and activated the CaSR using cinacalcet as above. The protocols include *1)* normal calcium media containing 1.2 mM ionized free calcium as control group, *2)* normal calcium media plus 5 μM U73122, *3)* normal calcium media with 1 μM of the CaSR agonist cinacalcet, and *4)* normal calcium media with 1 μM cinacalcet plus 5 μM of U73122. Cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.

*PLC inhibition with U73122 (n = 10).* To study if renin inhibition resulting from CaSR activation is dependent upon the PLC/IP_{3} pathway, we incorporated the PLC inhibitor U73122 (Amer, Thousand Oaks, CA) (27, 61) into our protocol, and activated the CaSR using the calcimimetic cinacalcet (44). The protocols included *1)* normal calcium media containing 1.2 mM ionized free calcium as control group, *2)* normal calcium media plus 5 μM U73122, *3)* normal calcium media with 1 μM of the CaSR agonist cinacalcet, and *4)* normal calcium media with 1 μM cinacalcet plus 5 μM of U73122. Cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.
μM cinacalcet, and 4) normal calcium media plus a combination of cinacalcet plus Ryanodine. Cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.

**IP₃ and ryanodine receptor inhibition** *(n = 10).* To study if the effects obtained with both the IP₃ and the RyR inhibitors after CaSR activation was additive, we incubated JG cells with the IP₃ inhibitor 2-APB (13) and with ryanodine, and activated the CaSR using cinacalcet as above. The protocols incubating JG cells included 1) normal calcium media containing 1.2 mM ionized free calcium as control group, 2) normal calcium media plus 1 μM cinacalcet, 3) normal calcium media with 1 μM cinacalcet plus 100 μM 2-APB, 4) normal calcium media with 1 μM cinacalcet plus 100 μM ryanodine, and 5) normal calcium media with 1 μM cinacalcet plus 100 μM 2-APB plus 100 μM ryanodine. Cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.

**Gₛ in the CaSR-Mediated Inhibition of Renin Release**

**Gₛ-protein coupled receptor inhibition with pertussis toxin (PTX)** *(n = 10).* To study whether renin inhibition after activation of the CaSR is coupled to the Gₛ protein, we incorporated 100 ng/ml of PTX (EMD Chemicals, Philadelphia, PA) as has been previously shown to inhibit Gₛ in isolated JG cells (37) and other cells in vitro (1, 34, 35, 37, 47, 52) into our protocols, and activated the CaSR using cinacalcet as above. In protocols incubating JG cells, we first pretreated cell for 3 h with either 100 ng/ml PTX or its vehicle DMSO, 1/1000 dilution) to block Gₛ. Then experiments were run over 2 h as above. The groups included 1) normal calcium media plus DMSO vehicle as a control, 2) normal calcium media with 100 ng/ml PTX, 3) normal calcium media with DMSO plus 1 μM cinacalcet, and 4) normal calcium media with DMSO plus 100 ng/ml PTX and 1 μM cinacalcet (52). Cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.

**PKA in the Calcium-Mediated Inhibition of Renin Release**

**PKA inhibition with Rp-8-CPT-cAMPS (CPT-cAMP)** *(n = 9).* It is well-established that the downstream target of JG cell cAMP formation is protein kinase A (PKA) activation (18, 36). We wanted to show that the calcium-mediated stimulation of renin release was also due to PKA. To do this we used an intracellular calcium chelator, BAPTA-AM (Sigma, St. Louis, MO), which we and others have shown increases renin secretion due to decreased intracellular calcium (40, 45, 46). This was coupled with the PKA inhibitor Rp-8-CPT-cAMPS (Sigma) (31, 53). The protocols using our isolated JG cells included 1) normal calcium media containing 1.2 mM ionized free calcium as a control, 2) normal calcium media plus 100 μM BAPTA-AM, 3) normal calcium media plus 100 μM CPT-cAMP (31), and 4) normal calcium media plus a combination of BAPTA-AM plus CPT-cAMP. In each protocol, cells were incubated for 2 h, after which the media was collected for determination of renin release and then the cells harvested for determination of total JG protein.

**Assay and Analysis**

**Renin release.** After 2 h, JG cell incubation medium was drawn off, centrifuged, and the supernatant recovered for the assay of the activity of renin released, as determined by angiotensin I generation. Samples were incubated for 3 h with the addition of excess rat angiotensinogen as substrate. The sample renin consumed less than 15% of exogenous substrate to ensure the enzymatic reaction remained in first-order kinetics. Angiotensin I generation was assayed using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) as previously described (45, 47). The incubation of media for angiotensin I generation is done using 0.1 mg phenylmethylsulfonylfluoride (PMSF) to inhibit protease activity. Values for renin concentration (μg ANG I generated-ml sample⁻¹·h incubation⁻¹) were corrected for JG cell total protein and are presented hereafter as micrograms ANG I per milliliter per hour per milligram protein.

**cAMP content.** After the incubation medium was removed for renin determination, JG cells were harvested for generally releasing the culture wells with 100 μl of PBS containing 1 mM IBMX plus 100 μl of 50% methanol. The cAMP content was determined from the harvested cells using an RIA kit (Biomedical Technology, Stoughton, MA). Values were corrected for JG cell total protein and expressed as picomoles per milligram protein.

**Protein concentration.** All determinations of renin released were corrected by the total JG cell protein. The protein concentration in JG cell lysates recovered from the plates was determined using the Coomassie plus Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions, as previously described (45, 46).

**Statistical analysis.** All data were derived and analyzed from paired control and experimental permutations in primary cultures from the same tissue pool obtained on a single day (each representing n = 1). Changes in renin release compared with controls were evaluated using ANOVA for repeated measures with a Bonferroni post hoc test, or a paired t-test where appropriate. We considered a P value <0.05 to be significant. In the figures, for the sake of simplicity, all statistically significant changes are represented as P < 0.05, while the actual P values are presented in the text of RESULTS. The n values are presented in each section of MATERIALS AND METHODS. Because of the documented seasonal variability in basal and stimulated renin release with in vitro preparations (10), all analyses are paired with their controls, and we have expressed our data in the figures as a percent of control for the sake of uniformity. The actual data are presented in the text, and all analysis is run using the actual data and not percents.

**RESULTS**

**Gₛ in the CaSR-Mediated Inhibition of Renin Release**

**CaSR inhibition with Ronacaleret (Fig. 1).** CaSR inhibition with Ronacaleret alone did not change basal renin release compared with controls in 0.9 mM calcium media (1.20 ± 0.20 to 1.27 ± 0.34 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹), and cAMP values also remained unchanged (1.18 ± 0.19 to 1.04 ± 0.18 pmol/mg protein). Incubation of the JG cells with the 1.5 mM calcium decreased basal renin release by 33% to 0.81 ± 0.12 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹ (P < 0.01 vs. control) and cAMP by 28% to 0.84 ± 0.15 pmol/mg protein (P < 0.05 vs. control) as expected. When JG cells were incubated in the 1.5 mM calcium media with Ronacaleret, the high-calcium-mediated inhibition of renin release was completely reversed (0.15 ± 0.18 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹), as was the decrease in cAMP content (0.98 ± 0.24 pmol/mg protein). Thus extracellular (media) calcium-mediated inhibition of renin seems to operate completely through JG cell CaSR.

**PLC inhibition with U73122 (Fig. 2).** The PLC inhibitor U73122 alone did not change basal renin release compared with control (0.40 ± 0.04 to 0.39 ± 0.04 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹). Incubation of the JG cells with the CaSR agonist cinacalcet decreased basal renin release by 44% to 0.22 ± 0.03 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹ (P < 0.01 vs. control), as expected. When JG cells were incubated with both cinacalcet plus U73122, PLC inhibition completely reversed the inhibition of renin by CaSR activation, returning renin to control values (0.39 ± 0.03 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹, P < 0.005 vs. cinacalcet alone). Thus PLC activa-
CaSR signaling via PLC/IP3 inhibits renin

IP3 inhibition of renin release (Fig. 3). Incubation of the JG cells with the CaSR agonist cinacalcet decreased basal renin release by 44% to 0.46 ± 0.05 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹ (P < 0.01 vs. control). Thus IP3 activation is an important component of the postreceptor pathway of the CaSR-mediated decrease in renin release from JG cells.

RyR in the CaSR-Mediated Inhibition of Renin Release

Immunolabeling of RyR in JG cells. We used a ryanodine antibody to immunolabel and confocal microscopy to detect the RyR in our primary culture of JG cells grown on cover slips. Figure 4 shows RyR localizing in two JG cells (shown in red, Fig. 4, B and D). The same cell was positively labeled for renin (shown in green, Fig. 4, A and C). Thus our isolated JG cells contain the RyR.

RyR inhibition (Fig. 5). Inhibition of the RyR with high concentrations of ryanodine did not change basal renin release compared with control (from 0.14 ± 0.01 to 0.17 ± 0.02 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹). Incubation of the JG cells with the CaSR agonist cinacalcet decreased basal renin release by 47% to 0.09 ± 0.01 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹ (P < 0.01 vs. control). When JG cells were treated with both cinacalcet plus ryanodine, renin release returned to control values (0.14 ± 0.01 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹). Thus ryanodine receptor activation appears to also participate in the CaSR-mediated decrease in renin release from JG cells.

IP3 and ryanodine receptor inhibition (Fig. 6). Incubation of JG cells with the CaSR agonist cinacalcet decreased basal renin release by 39% from 0.26 ± 0.04 to 0.13 ± 0.02 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹ (P < 0.05 vs. control). When JG cells were treated with both cinacalcet plus the IP3 inhibitor 2-APB and cinacalcet plus ryanodine, renin release returned to control values (0.31 ± 0.12 and 0.26 ± 0.05 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹, respectively), similar to results in Figs. 3 and 5. Incubation of the cells combining cinacalcet, ryanodine, and 2-APB together also completely reversed the Ca-mediated inhibition of renin release (0.33 ± 0.10 μg ANG I·mL⁻¹·h⁻¹·mg protein⁻¹), but this was not different from the responses by either inhibitor to reverse cinacalcet’s effect. Thus activation of the CaSR leads to IP3 and a RyR activation, but these appear not to be additive, suggesting they occur in series.
Gi in the CaSR-Mediated Inhibition of Renin Release

Gi-protein coupled receptor inhibition with PTX (Fig. 7). PTX inhibition of Gi appeared to decreased renin release, but this was not significant (0.68 ± 0.21 to 0.35 ± 0.10 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹). Incubation of the JG cells with the CaSR agonist decreased basal renin release by 35% to 0.31 ± 0.10 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹ (P < 0.05), as expected. When JG cells were incubated with both PTX plus cinacalcet, renin release remained significantly lower than controls (0.24 ± 0.09 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹, P < 0.05), and further was also significantly reduced compared with cinacalcet treatment alone (P < 0.05). Thus Gi activation does not appear to be involved in the postreceptor pathway of CaSR-mediated inhibition of renin release from JG cells.

PKA in the Calcium-Mediated Inhibition of Renin Release

PKA inhibition with Rp-8-CPT-cAMPS (CPT-cAMP) (Fig. 8). Calcium chelation with BAPTA-AM significantly increased renin release by 39% (0.63 ± 0.09 to 0.87 ± 0.15 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹) (P < 0.05), as expected (45, 46). Incubation of the JG cells with the PKA inhibitor, CPT-cAMP, did not change basal renin release (0.59 ± 0.09 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹). However, when JG cells were incubated with both CPT-cAMP and BAPTA-AM, renin release was not different from either controls or CPT-treated JG cells (0.55 ± 0.09 μg ANG I·ml⁻¹·h⁻¹·mg protein⁻¹). Thus renin stimulation mediated by decreased intracellular calcium is also PKA mediated.

DISCUSSION

In our studies of the CaSR on the JG cell, we found that similar to the CaSR in the parathyroid, activation of this

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**Fig. 4.** Immunofluorescence and confocal microscopy in two single JG cells (top and bottom) using 2 antibodies: one specific for renin (green; A and C) to confirm that each is a JG cell, and another specific for RyR (red; B and D). The JG cells are positive for both. Magnification: 100×.

**Fig. 5.** Renin release from JG cells under basal conditions (control, 1.2 mM media calcium) or after incubation with high concentrations of ryanodine (Ryan) to block the ryanodine receptor. These were run with or without the CaSR agonist cinacalcet. *P < 0.05 vs. control.
G-coupled receptor leads to stimulation of PLC, producing IP₃, leading to calcium-mediated inhibition of cyclic AMP formation and retarding renin release. We also found that this calcium-mediated pathway was enhanced by activation of the RyR. Finally, unlike the parathyroid, we did not find any evidence for a role of the Gᵢ pathway in JG cells mediating CaSR inhibition of renin release.

In the parathyroid glands, the CaSR plays an important role in calcium homeostasis, monitoring serum calcium and signaling for suppression of parathyroid hormone (48) secretion in response to elevated serum calcium. Activation of this Gq protein-coupled receptor by extracellular calcium results in inducing PLC, leading to the generation of IP₃ which binds to an inositol-P₃ receptor on the membrane of the endoplasmic reticulum, opening a calcium channel and releasing calcium from intracellular stores in this organelle (3, 23, 38, 49, 56).

Our data show that the PLC/IP₃ pathway is important in controlling CaSR-activated regulation of renin release from the JG cells, consistent with this pathway in the parathyroid. Previously, using a model of isolated vascular smooth muscle cells, Gq activation has also been reported to lead to calcium-mediated reduction of cyclic AMP formation through suppression of adenyl cyclases 5 and 6 (57). In the kidney, the CaSR has been identified in the proximal tubules, endothelial cells, cortical thick ascending limb cells, distal convoluted tubule, and cells of the collecting duct. We have documented its presence in the JG cells, both in vivo (6) and in vitro (44). Our data with the CaSR blocker Ronacaleret confirm that the calcium-mediated signaling we report is in fact due to activation of the CaSR, as blocking the CaSR completely eliminated calcium-mediated inhibition of renin release. We have previously described the CaSR on JG cells as a conduit linking the calcium concentration in the extracellular renal cortical interstitium to the intracellular concentrations as a pathway to regulate renin release (6, 44). This occurs through increasing intracellular calcium, which suppresses the activity of the calcium-inhibitable adenyl cyclase isoform-5 (45), possibly also isoform-6 (32), and also by activating the calcium-stimulated phosphodiesterase 1C (43). Thus increased intracellular calcium leads to suppression of synthesis and enhanced degradation of cyclic AMP, the dominant cyclic nucleotide second messenger regulating renin secretion (20, 36). Previous studies describing the calcium-mediated regulation of both cyclic AMP and renin (6, 32, 43–46) are consistent with this model of the CaSR regulating intracellular calcium. To this we now describe that the PLC/IP₃ pathway is the critical postreceptor means by which this happens.

In addition to PLC/IP₃, intracellular calcium signaling can open store-operated calcium channels, and this can be amplified by activation of the RyR (24). Wong et al. (59) have described a role for the ryanodine receptors in parathyroid cells in regulating parathyroid hormone (PTH) secretion via the CaSR. Fellner and Arendshorst (24, 25, 55) have shown that the RyR exists in the renal afferent arteriolar vascular smooth muscle, which is contiguous with the JG cells. The JG cells share common features with the vascular smooth muscle cells of the afferent, especially in the terminal segment of the arteriole, and may be derived from common progenitor cells (51). RyR activation in the afferent arteriole increases intracellular calcium and amplifies renal vasoconstriction. The RyR amplifies the IP₃-mediated calcium release from the ER in a
process called calcium-induced calcium release (12). Using fluorescent-tagged antibodies for renin and for the RyR, we show both colocalizing in our preparation of JG cells (39). While ryanozine can activate the RyR at nanogram concentration, we used microgram concentrations that have been shown to inhibit the RyR (25). We found RyR inhibition, similar to PLC and IP3 inhibition, completely reversed the CaSR, calcium-mediated inhibition of renin release from JG cells. When JG cells were incubated with both the ryanozine inhibitor and the IP3 inhibitor after activation of the CaSR, the effect in renin release was not additive. Thus our data provide both immunohistochemical and functional evidence that the RyR does exist in the JG cells, and further it is a key component as a final element in the PLC/IP3 postreceptor pathway releasing intracellular calcium in response to activation of the CaSR on the JG cell.

While in our model we are unable to directly measure intracellular calcium we believe the primary source of increased intracellular calcium is from the endoplasmic reticulum. Calcium release from the endoplasmic reticulum is known to be mediated by PLC-IP3 activation, as well as a ryanodine activation (3, 23, 38, 49, 56). Because we have previously shown that the target adenylyl cyclase (AC-V) is localized on the renin-containing granules, it suggests the compartment into which ionized free calcium is being released is the cytoplasm surrounding the granules. However, the present protocols do not allow us to specifically identify calcium in particular intracellular compartments.

CaSR activation in the parathyroid gland has been proposed to act via activation of an inhibitory G receptor-coupled protein (16, 19, 48). The inhibitory G proteins (G) are the most highly expressed and predominant family of G proteins, including four isoforms that share great homology, and are widely distributed in many tissues (58). Inhibitory G proteins typically act to inhibit adenylyl cyclase activity leading to diminished cAMP production (50, 60). We wanted to test if this possible pathway was an alternative, or possibly in addition to the PLC/IP3 activation pathway coupled to intracellular calcium. To do this we employed the non-isoform-selective G inhibitor PTX (34, 52). We did not find any amplification of renin release when we inhibited Gs, and in fact if anything it was decreased. While PTX is a rather nonspecific and toxic tool, the total absence of any positive effect on renin suggests, dissimilar to the parathyroid, Gs is not playing a significant role in CaSR-mediated renin inhibition in the JG cell.

We wanted to confirm that the changes in calcium-mediated renin were channeling through the cAMP-stimulated PKA pathway, as has been previously documented for renin stimulation (28, 30, 31). cAMP exerts its influence on secretion of active renin via PKA. PKA phosphorylates proteins that initiate the release of renin from the storage granules within the JG cell. PKA inhibitors eliminate either renin secretion or changes in JG cell membrane capacitance (29, 30) as a surrogate for renin release. However, none of these studies have targeted the effect on calcium-mediated renin secretion. It was important to show the calcium-mediated regulation of renin we refer to was mediated by PKA and not due to some alternative undescribed pathway involving calcium. To do this we used intracellular calcium chelating to directly reduce intracellular calcium, a technique we have previously shown to cause cAMP formation and renin release from JG cells (45, 46). As expected, we found that reducing intracellular calcium led to increased renin release from the JG cells, and this response was completely blocked by inhibiting PKA. This provides further evidence that the calcium-mediated control of renin in the JG cell, beginning with CaSR activation and ending in cAMP-mediated PKA activation stimulating renin release, is the same pathway described for the regulation of renin secretion (11).

So can we extrapolate these in vitro data into a meaningful role of calcium in the regulation of renin in the whole kidney? It is clear that calcium-mediated inhibition of renin by vasoconstrictors like angiotensin II, acting through the AT-1 receptor, plays a direct role in sodium homeostasis and blood pressure control (reviewed in 4 and 11). However, recent data from our laboratories (5, 6) suggest the CaSR on the JG cell is coupled to PTH-mediated distal tubular calcium reabsorption. The renal cortical interstitium serves as the medium to which the CaSR on the JG cell responds in vivo (5). This suggests CaSR-mediated renin may be coupled to calcium homeostasis rather than sodium, and may be linked to preserving blood pressure rather than inducing hypertension (4, 7).

Overall, our goal was to describe the postreceptor pathway by which JG cell CaSR activation led to the calcium-mediated control of renin release. Much like the parathyroid gland, we report that CaSR activation led to a G protein-coupled induction of the PLC/IP3 signaling pathway. In the JG cell this resulted in calcium-mediated inhibition of renin release. Presumably this is due to IP3 release of calcium from intracellular stores, and the effect of previously documented increases in intracellular calcium on cAMP synthesis and degradation (32, 43–46). We also found the release of calcium from intracellular stores is coupled to the ryanozine receptor, which presumably augments or amplifies the IP3 signal to enhance the release of intracellular calcium. Finally, in contrast to the parathyroid, we did not find a role for Gs-mediated inhibition in the CaSR signaling cascade. Importantly, the activation of the JG cell CaSR is not in itself a direct regulatory pathway for renin secretion, but appears to change the baseline by modifying the activity of the enzymes (AC-V, PDE1C) that are the targets of the classical renin stimuli.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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