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Modulation Of Renin Secretion By Renal Cortical Interstitial Calcium

Douglas K. Atchison
Wayne State University,

MODULATION OF RENIN SECRETION BY RENAL CORTICAL INTERSTITIAL CALCIUM

by

DOUGLAS K. ATCHISON

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DEDICATION

To my family for their love and support.

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PREFACE

General Background

Renin is a proteolytic enzyme, produced in and secreted from the juxtaglomerular cells of the afferent arteriole.[18] Renin is the rate-limiting enzyme of the renin-angiotensin system. Renin cleaves the leucine-valine bond in circulating angiotensinogen, converting it to angiotensin I.[26] Angiotensin I is later converted by angiotensin converting enzyme to Angiotensin II, and Angiotensin II is the major effector molecule of the renin-angiotensin system. The production of renin (and subsequently angiotensin II) is important for the regulation blood pressure, circulating volume and renal homeostasis. This is illustrated by the fact that renin knockout mice have impaired viability, and those that do survive are profoundly hypotensive, are unable to concentrate urine, and have impaired renal development.[79] Renin is also important in the pathogenesis of hypertension. Approximately 70% of patients with hypertension have inappropriately elevated plasma renin levels.[13] Inhibitors of the renin-angiotensin system (including direct inhibitors of renin) significantly decrease blood pressure and are a mainstay of anti-hypertensive therapy. Thus, factors that affect renin secretion and regulation are of both scientific and clinical interest. Renin can be quantified by several methods *in vivo*. The most common method to quantitate renin is known as plasma renin activity (PRA).[14] PRA is defined as the amount of Angiotensin I generated by plasma renin from angiotensinogen endogenous to the plasma. PRA represents renin secreted from the JG cells, as bilaterally-nephrectomized patients or rats have their PRA levels fall to essentially undetectable levels.[38,52]

PRA is decreased by elevated plasma calcium (Ca).[40,41,74] This has been known for over 30 years, but the mechanism by which it occurs is unknown. Recently, it has been demonstrated that many of the effects of plasma Ca are mediated by a ubiquitously expressed calcium-sensing receptor (CaSR).[12] The CaSR is a seven-transmembrane domain, G-protein-coupled receptor

that transmits changes in extracellular Ca into changes in intracellular signaling. The CaSR is most highly expressed in tissues that are important for Ca metabolism, such as the parathyroid gland and kidney. The concerted action of the CaSR in these various tissues is to homeostatically lower plasma Ca in response to hypercalcemia; the parathyroid-expressed CaSR decreases parathyroid hormone (PTH) secretion in response to elevated plasma Ca, and the CaSR expressed in the kidney promotes the excretion of Ca in response to hypercalcemia. Since the CaSR inhibits PTH secretion from the chief cells of the parathyroid gland, it was hypothesized that the CaSR would mediate the inhibition of renin release by elevated extracellular Ca. In support of this notion, the CaSR is expressed by juxtaglomerular (JG) cells *in vitro* in the renin-containing granules, and CaSR agonists inhibit renin release *in vitro*.[53,54] However, whether the CaSR mediates the high-Ca inhibition of PRA remains to be determined. The intracellular mechanisms for how the CaSR inhibits renin release *in vitro* are well defined. Cyclic adenosine monophosphate (cAMP) is the major stimulatory second messenger for renin release. The CaSR inhibits the production of intracellular cAMP by decreasing the activity of a calcium-inhibitable isoform of adenylate cyclase (adenylate cyclase-V),[55,56] and also by stimulating the activity of a Ca-activated, cAMP-degrading phosphodiesterase, PDE-1.[53] Thus, the net effect of the CaSR is to decrease JG cell cAMP and inhibit renin release.

We anticipate that elevated renal cortical interstitial Ca during hypercalcemia will be what inhibits PRA. *In vivo*, the renin secreting JG cells are bordered luminally by fenestrated endothelium. It is possible that Ca could pass through these fenestrae to inhibit renin release directly from the JG cell, but seems unlikely as the endothelial cells are coupled to JG cells *via* the tight junction channels called connexins.[73] Alternatively, the basolateral border of the JG cells is freely bathed in the renal cortical interstitium. As such, changes in renal cortical interstitial Ca could affect PRA as well. Increasing plasma Ca increases renal cortical interstitial

Ca.[48] We anticipate increased interstitial Ca will act on the CaSR to decrease PRA. A brief review of renal Ca handling will highlight which transport processes we anticipate would most likely be responsible for the response of PRA to renal cortical interstitial Ca.

Ca is found in predominantly two forms in the plasma: calcium that is bound to proteins (predominantly albumin) which is not filtered at the glomerulus, and filtered calcium. Free, ionized calcium represents 80% of the filterable calcium, while the remainder is complexed to anions.[80] Free, ionized Ca is the form of Ca which interacts with the CaSR. Approximately 97-99% of the filtered Ca load is reabsorbed by the kidney. Of the filtered load of Ca, the proximal tubule is responsible for 50-70% of its reabsorption.[80] Ca reabsorption in the proximal tubule closely follows water and NaCl reabsortion.[80,1] The reabsorption of Ca occurs through the paracellular diffusion, presumably through claudin-2, a tight-junction protein.[2,11,21] The thin descending limb does not reabsorb Ca.[80] The thick ascending limb (THAL) of the Loop of Henle reabsorbs about 15% of the filtered load of Ca.[80] The reabsorption of Ca occurs down a concentration gradient and is enhanced by the lumen positive electrical potential.[9, 80] The lumen positive potential of the THAL is generated by the reabsorption of Cl^-, and also by K secreted into the lumen through the ROMK channel.[30] This increases the positivity of the lumen, which increases the driving force for passive, paracellular Ca reabsorption.[9] Ca reabsorption occurs though the paracellular channel, claudin-16.[64] The distal and connecting tubules are responsible for 10-15% of Ca reabsorption.[80] In these nephron segments, Ca is reabsorbed transcellularly through TRPV5.[33] PTH exerts its main hypocalciuric effects in these segments by stimulating the production and activity of TRPV5.[17,19,32,33,69] Calbindin-D28K, a Ca-binding protein, is necessary for maintaining the high luminal to intracellular Ca gradient, allowing for Ca to flow down its concentration gradient into the distal tubule epithelia.[32] Additionally, Vitamin D increases TRPV5 expression.[32] While the amount of Ca reabsorbed in this segment is relatively

small, the distal tubule has a large effect on how much Ca is excreted, since much of the Ca reabsorption is under hormonal control. The collecting duct is responsible for about 1% of Ca reabsorption, which occurs through TRPV6.[80]

The CaSR also plays an important role in renal Ca handling.[12] The CaSR is expressed almost ubiquitously throughout the nephron: It has been detected in the proximal tubule, thick ascending limb, distal tubule and the cortical and medullary collecting ducts.[60] It is found apically in both the proximal tubule and the inner medullary collecting duct.[60] The CaSR is found basolaterally in the thick ascending limb, distal tubule and cortical collecting duct.[60] In the proximal tubule, high tubular Ca inhibits the Na-K-ATPase transporter, ostensibly by acting on the CaSR.[28] This may contribute to the diuretic effects of hypercalcemia. In the THAL, activation of the basolateral CaSR decreases the secretion of K into the tubular lumen, which decreases the positive luminal voltage potential.[12] This decreases the driving force for paracellular Ca reabsorption and increases Ca excretion.[12] Also, in the THAL, activation of the CaSR inhibits cAMP production, and may inhibit Na-K-2Cl (NKCC-2) transporter trafficking, contributing to its diuretic effects.[20] How the CaSR affects transport in the distal tubule and collecting duct is not well defined.

PTH is an 84 amino acid peptide secreted from the chief cells of the parathyroid gland. PTH is released into the plasma in response to hypocalcemia. PTH increases plasma Ca predominantly by increasing Ca resorption from bone and also by increasing the renal reabsorption of Ca. There are 4 sites along the nephron where PTH may affect Ca reabsorption: the glomerulus, the proximal tubule, thick ascending limb, and distal tubule. PTH acts to increase plasma Ca by inhibiting the filtering of Ca at the glomerulus by decreasing the filtration coefficient (K_f).[36] In contrast, PTH paradoxically inhibits proximal tubule Ca reabsorption by inhibiting the Na-H exchanger (NHE) in the proximal tubule.[16] Because Ca reabsorption is

coupled to the reabsorption of Na in the proximal tubule,[1,80] this inhibition of NHE decreases Ca reabsorption in this nephron segment.[1] However, distal Ca reabsorption compensates for this. PTH stimulates Ca reabsorption in the cortical THAL.[22,43] PTH-stimulated Ca transport in the thick ascending limb occurs through a passive mechanism *via* paracellular pathways.[10,37,77] It is not known if the PTH-mediated paracellular reabsorption pathway for Ca in the thick ascending limb occurs through claudin-16. The best characterized effects of PTH on Ca reabsorption are in the distal tubule.[17] Here, PTH stimulates the active transport of Ca *via* multiple mechanisms. First, PTH stimulates the synthesis and activity of the distal tubule Ca transporter, TRPV5.[19,32,69] The low intracellular to extracellular Ca gradient is maintained by the Ca-binding protein, Calbindin-D28K, in the distal tubule epithelia. Ca is pumped into the renal interstitium by the Na-Ca antiporter or Ca-ATPases. PTH stimulates the expression of the Na-Ca antiporter, which is responsible for approximately 75% of the basolateral Ca transport.[33] The net effect of PTH on all of these reabsorptive processes in different nephron segments is to stimulate Ca reabsorption and inhibit urinary Ca excretion.[33] This is reflected in the effects of PTH on renal cortical interstitial Ca: PTH increases renal cortical interstitial Ca.[48] As such, PTH is integral for increasing renal cortical interstitial Ca mainly due to it stimulatory effects on Ca reabsorption in the distal nephron.

As such, we anticipate the following: since the CaSR has been shown to inhibit renin release *in vitro*, we anticipate that it should inhibit PRA *in vivo* as well. Furthermore, we anticipate that hypercalcemia will also inhibit PRA by acting on the CaSR. We anticipate that hypercalcemia will decrease PRA *via* increased renal cortical interstitial Ca. Because PTH increases renal cortical interstitial Ca, we expect that hypercalcemia will increase renal cortical interstitial Ca in a PTH-dependent manner. Thus, the overall hypothesis that we propose is; that hypercalcemia inhibits PRA *via* PTH-mediated increases in renal cortical interstitial Ca that act

on the CaSR triggering Ca-mediated inhibition of renin secretion.

CHAPTER 1: ACUTE ACTIVATION OF THE CALCIUM-SENSING RECEPTOR INHIBITS PLASMA RENIN ACTIVITY *IN VIVO*

ABSTRACT

In vitro, the renin-secreting juxtaglomerular cells express the calcium-sensing receptor, and its activation with the allosteric CaSR agonist, Cinacalcet, inhibits renin release. To test if the activation of calcium-sensing receptor similarly inhibits plasma renin activity *in vivo,* we hypothesized that the calcium sensing receptor is expressed in juxtaglomerular cells *in vivo*, and acutely-administered Cinacalcet would inhibit renin activity in anesthetized rats. Since Cinacalcet inhibits parathyroid hormone, which may stimulate renin activity, we sought to determine if Cinacalcet inhibits renin activity by decreasing parathyroid hormone. Lastly, we hypothesized chronically administered Cinacalcet would inhibit basal and stimulated renin in conscious rats. Calcium sensing receptors and renin were localized in the same juxtaglomerular cells using immunofluorescence in rat cortical slices fixed *in vivo*. Cinacalcet was administered acutely *via* intravenous bolus in anesthetized rats and chronically in conscious rats by oral gavage. Acute administration of Cinacalcet decreased basal renin activity from 13.6 ± 2.4 to 6.1 ± 1.1 ngAngI/ml/hr ($p<0.001$). Likewise, Cinacalcet decreased furosemide-stimulated renin from 30.6 ± 2.3 to 21.3 ± 2.3 ngAngI/ml/hr ($p<0.001$). In parathyroidectomized rats Cinacalcet decreased renin activity from 9.3 ± 1.3 to 5.2 ± 0.5 ngAngI/ml/hr ($p<0.05$) similar to sham-operated controls (13.5 ± 2.2 to 6.6 ± 0.8 ngAngI/ml/hr, $p<0.05$). Chronic administration of Cinacalcet over 7 days had no significant effect on plasma renin activity under basal or stimulated conditions. In conclusion, calcium sensing receptors are expressed in juxtaglomerular cells *in vivo*, and acute activation of these receptors with Cinacalcet inhibits plasma renin activity in anesthetized rats, independent of parathyroid hormone.

INTRODUCTION

The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor that senses changes in extracellular calcium (Ca) and transmits these into parallel alterations in Ca-mediated intracellular signaling.[31] The CaSR is expressed in the parathyroid and thyroid glands, intestines, and kidney.[12,60] Stimulation of the CaSR inhibits parathyroid hormone (PTH) secretion, decreases plasma Ca and increases urinary Ca excretion.[12] Calcimimetics, such as Cinacalcet-HCl (Sensipar, Amgen Inc., Thousand Oaks, CA), increase the sensitivity of the CaSR to the ambient concentration of extracellular Ca.[31] This leads to enhanced CaSR-mediated intracellular signaling at a given concentration of extracellular Ca. Calcimimetics decrease plasma PTH and plasma Ca. Calcimimetics are used clinically to treat both secondary hyperparathyroidism and parathyroid carcinomas.[31]

Renin is secreted from the juxtaglomerular (JG) cells in the afferent arteriole of the kidney and cleaves angiotensinogen to angiotensin I.[7] Angiotensin I is the biological precursor for the vasoconstrictor agent, angiotensin II. The renin-angiotensin system is critical for blood pressure and volume homeostasis, and its inhibition is one mainstay of anti-hypertensive therapy. Furosemide stimulates renin secretion predominantly by stimulating nitric oxide and prostaglandin production from the macula densa.[4,46]

Recently, we have shown that the CaSR is expressed in primary cultures of isolated, renin-secreting mouse JG cells, and CaSR activation with the allosteric CaSR agonist, Cinacalcet, inhibits renin release *in vitro*.[54] Cinacalcet acts by decreasing the EC_{50} of the CaSR for extracellular Ca and has a terminal half-life *in vivo* of 30-40 hrs.[15] It has been reported that Cinacalcet may acutely inhibit PRA *in vivo*,[45] but whether Cinacalcet can inhibit PRA chronically is unknown. Also, since Cinacalcet inhibits PTH, and PTH has been reported to stimulate PRA,[66] we wanted to test if the inhibition of PRA by Cinacalcet was due to its ability

to decrease PTH. Thus, we hypothesized that the CaSR is expressed in JG cells *in vivo*, that acutely-administered Cinacalcet would inhibit PRA in anesthetized rats, but that this stimulus would be eliminated by parathyroidectomy. Finally, we hypothesized that chronically administered Cinacalcet would also inhibit PRA in conscious rats under basal and stimulated conditions.

MATERIALS & METHODS

Fixing the renal cortex *in vivo*: Male Sprague-Dawley rats were anesthetized using 125 mg/kg body weight thiobutabarbitol, (Inactin, Sigma) I.P. before having their abdominal cavities opened and their left kidneys flushed with 150 mmol/L NaCl by retrograde perfusion through the abdominal aorta. The kidney was then fixed *in situ* for 15 min *via* perfusion with 4% paraformaldehyde in buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate (pH 7.4). The kidney was removed and stored in 4% paraformaldehyde at 4° C until ready for slicing. The poles of the kidney were sliced off, and the fixed renal cortex was embedded in paraffin. 5 μm slices were mounted on microscope slides for immunofluorescence experiments.

Coimmunolabeling of renin and CaSR: Fixed, paraffin-embedded cortical slices were first de-paraffinized three times in Xylene, then hydrated gradually through graded alcohols: 100% ethanol (2 times), 95% ethanol, 70% ethanol and finally distilled water. All washes were for 5 min. Slices were permeabilized with 0.1% Triton-X100 for 10 min at 37° C. The CaSR was detected by incubating 1 hr at 37° C with a 1:40 dilution of the CaSR antibody (Affinity Bioreagents, Golden, CO) followed by incubating for 1 hr at 37° C with a 1:100 dilution of the secondary antibody (Alexa Fluor 488 goat antimouse IgG, Molecular Probes, Invitrogen Corp., Carlsbad, CA). Slides were then incubated for 1 hour at 37° C with a 1:25 dilution of an antibody raised in sheep against both rat and mouse renin (Innovative Research Inc., Novi, MI) followed by incubating for 1 hr at 37° C with a 1:100 dilution of secondary antibody (Alexa Fluor 568 goat

antisheep IgG, Molecular Probes). CaSR fluorescence was detected at 40X with a confocal laser scanning system (Visitech Intl., Confocal System, Sunderland, UK) set at 488 nm excitation with a 500 nm long-pass filter. The same settings were used to detect renin, except that 568 nm excitation and a 590 nm band pass filter were used.

Acute i*n vivo* protocol:

Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) weighing 250-350 g were fasted overnight but allowed free access to water. They were anesthetized using thiobutabarbital, 125 mg/kg body weight I.P. (Inactin, Sigma, St. Louis, MO) and placed on a heating pad to maintain constant body temperature. A tracheotomy was performed with PE-240 tubing to allow spontaneous breathing of room air. The femoral vein was catheterized with PE-50 tubing for infusion of 10 µl/min 0.9% NaCl. The femoral artery was catheterized with PE-50 tubing attached to a Statham pressure transducer (Viggo-Spectramed, Oxnard, CA), calibrated using a "Traceable" electronic manometer (Fisher Scientific, Pittsburgh, PA), and connected through an iWorx 118 A to D Signal Processor to a computer using iWorx Labscribe 2.065 data acquisition software (iWorx, Dover, NH) for continuous monitoring of mean arterial pressure (MAP).

After surgery, the rats received a supplemental bolus of 1.0 ml of 6% heat-inactivated bovine serum albumin (BSA, Sigma, St. Louis, MO) in normal saline and were allowed to equilibrate for 60 min. All blood samples taken were 300 µL in volume, and were replaced with an equal volume of 6% BSA. When the experiments were completed, the rats were euthanized by bilateral pneumothorax and aortic transection, and the kidneys decapsulated, excised and inspected for anatomical abnormalities.

These procedures were reviewed and approved by our Institutional Animal Care and Use Committee (IACUC) and adhere to the guiding principles in the care and use of experimental

animals and conducted in accordance with the national Institutes of Health (NIH) Guide for the Care and Use of laboratory Animals. Henry Ford Hospital's animal facility is AAALAC-approved.

Parathyroidectomy Procedure:

Rats were anesthetized using 50 mg/kg bodyweight Nembutal (Pentobarbital Sodium, Ovation Pharmaceuticals, Deerfield, IL), I.P. The surgical procedure was performed using aseptic techniques on a heating pad to maintain constant body temperature. The anterior portion of the rat neck was shaved and swabbed with betadine (Purdue Frederick, Norwalk, CT). A longitudinal incision on the anterior portion of the rat's neck was made, and the connective tissue and underlying muscle was teased apart with forceps. The parathyroid glands were isolated on the lateral, superior portion of the thyroid glands and were excised. The incision was then sutured and stapled closed and covered with colloidion iodine. Rats received 10 ml of subcutaneous sterile 0.9% NaCl immediately post-surgery to prevent dehydration. Sham surgeries were performed identically, except the parathyroid glands were left intact. Rats were used 24-48 hours post-surgery for acute protocols. Parathyroidectomized rats were excluded from analyses if their basal plasma PTH was at all detectable by assay.

***In vivo* protocols:**

I. The effect of acutely-administered Cinacalcet on basal PRA:

Group 1: *Cinacalcet 5 mg/kg*: When the rats finished recovering after the surgical procedure, blood was withdrawn for a basal PRA. The rats then received a 5 mg/kg I.V. bolus of Cinacalcet, dissolved in dimethylsulfoxide (DMSO) and diluted into 500 µl of 0.9% NaCl. Samples of blood were withdrawn for PRA at 15, 30 and 60 minutes post-Cinacalcet. Blood for measurement of parathyroid hormone (PTH) and plasma Ca was withdrawn at 60 minutes post-Cinacalcet after the final PRA sampling (n=7). Cinacalcet was purchased from the Henry Ford

Hospital Pharmacy, and was not provided by Amgen or any intermediary supplier.

Group 2: *Cinacalcet 1 mg/kg*: Protocols were run identically to group 1, except that the rats received only 1 mg/kg Cinacalcet I.V. (n=8).

Group 3: *vehicle*: Protocols were run identically to group 1, except that the rats received an equal volume of the DMSO vehicle without Cinacalcet (n=11).

II. The effects of acutely-administered Cinacalcet on plasma Ca and PTH over time:

Additional protocols were run identically to Protocol I, except that plasma PTH and Ca were measured basally, and at each of the15, 30 and 60 minutes time points post-Cinacalcet or vehicle bolus.

III. The effect of acutely-administered Cinacalcet on furosemide-stimulated PRA:

Group 1: *Cinacalcet 5 mg/kg + furosemide*: Rats were anesthetized and instrumented as described. After recovery from surgical instrumentation, while still under anesthesia, rats received an I.V. bolus of 5 mg/kg furosemide[1] (American Regent, Inc., Shirley, NY) and 30 minutes later blood was withdrawn to determine furosemide-stimulated PRA. Next, rats received a 5 mg/kg I.V. bolus of Cinacalcet. Blood was withdrawn for PRA measurements at 15, 30 and 60 minutes post-Cinacalcet. Blood for the measurement of PTH and plasma Ca was withdrawn at 60 minutes post-Cinacalcet after the final PRA sampling (n=8).

Group 2: *vehicle + furosemide*: Protocols were run identically to group 1, except that the rats received an equal volume of the DMSO vehicle without Cinacalcet (n=9).

IV. The effect of acutely-administered Cinacalcet in parathyroidectomized (PTX) rats:

Group 1: *Cinacalcet 5 mg/kg + PTX*: PTX rats received a 5 mg/kg I.V. bolus of Cinacalcet after having basal PRA, PTH and plasma Ca samples taken. PRA samples were taken at 15, 30 and 60 minutes post-Cinacalcet. Blood for measurement of parathyroid hormone (PTH) and plasma Ca was withdrawn prior to Cinacalcet administration and at 60 minutes post-

Cinacalcet after the final PRA sampling (n=4).

Group 2: *Cinacalcet 5 mg/kg + sham*: Experiments were run identically to group 1, using sham-operated rats (n=7).

V. Chronic protocols with Cinacalcet *via* oral gavage:

Chronic studies included both basal and stimulated PRA in unanesthetized, conscious rats. Rats with unstimulated PRA received 10 mg/kg bodyweight Cinacalcet *via* oral gavage. Cinacalcet was administered once per day in 500 µl of 3.3% glucose. Vehicle control rats received identical treatment with 3.3% glucose, but did not receive Cinacalcet. After 7 days of receiving Cinacalcet or the vehicle, blood was collected by decapitation for PRA and plasma Ca measurements. Cinacalcet has a half-life of 30-40 hours post-absorption.[15] Seven days was chosen because steady-state levels of the drug are achieved within this time frame.[15] Only the first 3 seconds of blood after decapitation were collected for PRA to ensure renin released by the baroreceptor mechanism did not contaminate the sample. Both the Cinacalcet and vehicle groups had an n=6.

In the study examining the chronic effect of Cinacalcet on stimulated PRA, 25 mg/kg Losartan Potassium (Sigma, St. Louis, MO) was delivered in 0.9% NaCl *via* oral gavage, while 20 mg/kg Furosemide (Hospira, Lake Forest, IL) was delivered *via* an I.P. injection.[4,6] Cinacalcet (20 mg/kg bodyweight) was delivered daily *via* oral gavage identically as above. Vehicle control rats received identical treatment, but did not receive Cinacalcet. All drugs were given once per day for 7 days before blood was collected *via* decapitation identically to rats with unstimulated PRA. Blood for plasma Ca and PTH measurements was also collected. The Cinacalcet group had an n=9, and the vehicle control group had an n=11.

Plasma Renin Activity (PRA): PRA was analyzed from 300 µl of femoral venous blood. Blood was centrifuged at 9800 x g for 6 min and the plasma was aspirated and stored at -20° C

until PRA was determined. Plasma renin activity was analyzed by generation of angiotensin I (Ang I/ hr/min) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) as previously described and according to the manufacturers instructions. The minimum resolution we can obtain with our assay is 0.13 ng Ang I/ml/hr.

Plasma Ca and PTH measurements: Measurements of plasma Ca and PTH were performed on femoral venous blood samples. Plasma Ca was measured using a NOVA-1 electrolyte analyzer (Nova Biomedical, Waltham, MA) while plasma PTH was measured using a commercial, rat PTH immunoassay kit (Alpco Diagnostics, Salem, NH) according to the manufacturer's instructions.

Statistics: Changes from baseline to post-treatment were analyzed using One-way Repeated-measures ANOVA with a Student-Newman-Keuls test for post-hoc analysis for multiple measurements, while single measurements post-baseline were measured using a paired *t*-test. Intergroup comparisons comparing multiple groups were performed using a One-way ANOVA with a Student-Newman-Keuls test for post-hoc analyses; while single intergroup comparisons were performed using a Student's unpaired *t*-test. For intergroup comparisons of PRA, the values were transformed to a percentage of their basal value. The appropriate intergroup test was used on these transformed values at their specific time points. Values $p < 0.05$ were considered statistically significant in all cases. All data are presented as mean ± SEM.

RESULTS

Coimmunolabeling of renin and CaSR: Figure 1 represents two cortical sections (A and B) labeled for the CaSR and renin. The left panels illustrate the expression of the CaSR (green), which is found throughout the proximal tubule as expected,[60] as well as the afferent arteriole (aa) next to the glomerulus (G), while the glomeruli were completely negative. The right panels show the expression of renin (red) within the same JG cells found in the afferent

arteriole. The immunolabeling demonstrates that the CaSR is localized in the renin-containing JG cells in the afferent arteriole in rat renal cortical slices fixed *in vivo*.

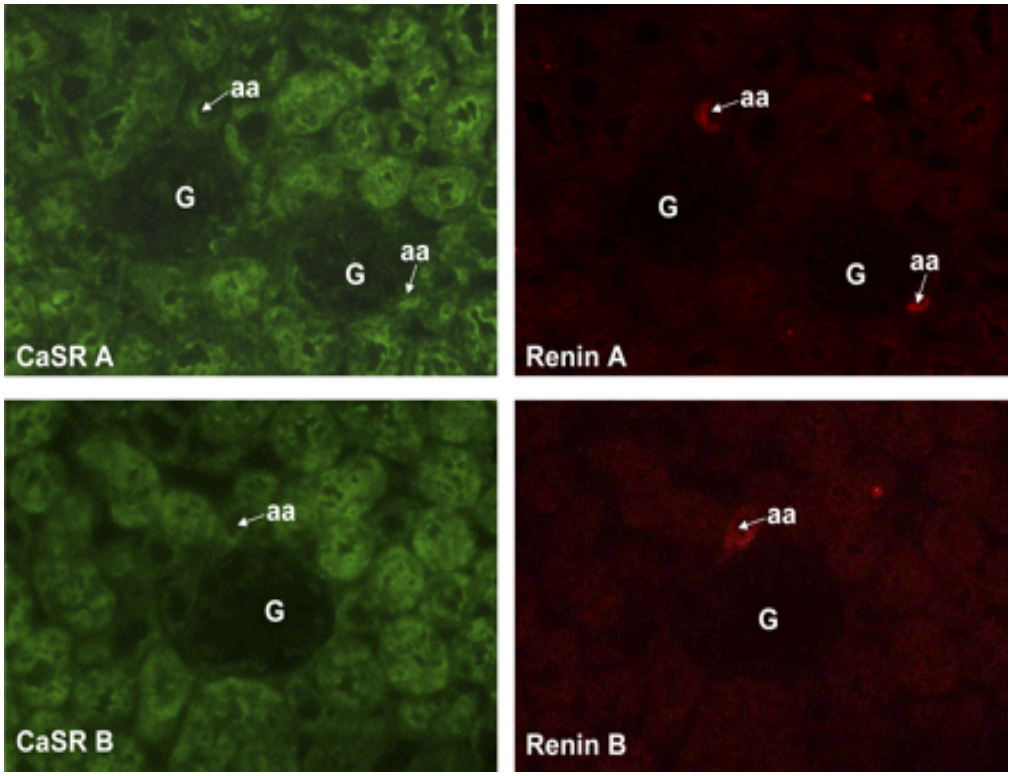


Figure 1: Fluorescent microscopy at 40X showing renin (red) is expressed with the CaSR (green) in the afferent arteriole in two different rat renal cortical slice images (A and B) fixed *in vivo*. aa: afferent arteriole, G: glomerulus.

I. The effect of acutely-administered Cinacalcet on basal PRA:

To test whether acute activation of the CaSR inhibited basal PRA *in vivo,* we treated anesthetized rats with the vehicle control, 1 mg/kg Cinacalcet or 5 mg/kg Cinacalcet. The basal PRA for the vehicle control group was 12.7 ± 2.7 ng Ang I/ml/hr and was unchanged at 15, 30 and 60 minutes post-vehicle (11.1 ± 2.6, 11.0 ± 2.7 and 10.5 ± 1.9 ng Ang I/ml/hr, respectively). The basal PRA in the 1 mg/kg Cinacalcet group was 16.2 ± 2.0 ng Ang I/ml/hr. 1mg/kg Cinacalcet significantly decreased PRA at 15, 30 and 60 minutes post-1 mg/kg Cinacalcet to 9.3 ± 1.4 (p<0.001), 8.8 ± 2.0 (p<0.001) and 10.4 ± 1.8 ng Ang I/ml/hr (p<0.001), respectively. The basal PRA in the 5 mg/kg Cinacalcet group was 13.6 ± 2.4 ng Ang I/ml/hr. 5 mg/kg Cinacalcet

significantly decreased PRA at 15, 30 and 60 minutes post-5 mg/kg Cinacalcet to 6.9 ± 1.2 (p<0.001), 6.1 ± 1.1 (p<0.001) and 8.2 ± 1.7 ng Ang I/ml/hr (p<0.001), respectively.

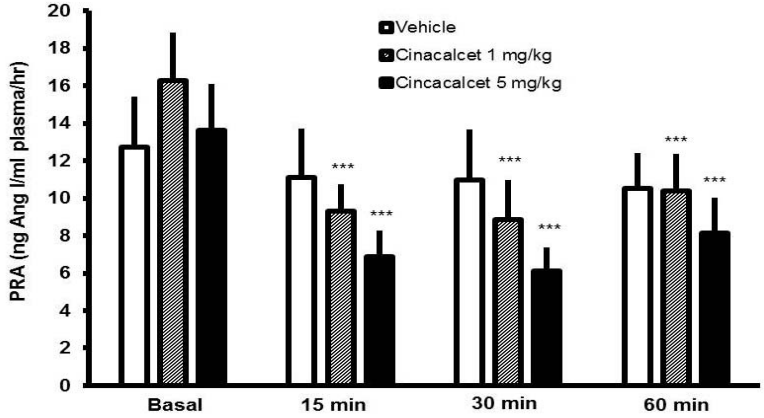


Figure 2: The acute effects of Cinacalcet on basal PRA in anesthetized rats. 5 mg/kg and 1 mg/kg Cinacalcet significantly decreased PRA at 15, 30 and 60 minutes post-treatment, respectively. PRA in the vehicle-treated group did not change. ***p<0.001 versus basal. N=7-11.

Basal MAP in the vehicle group 1 mg/kg and 5 mg/kg Cinacalcet-treated groups were 98 ± 2, 100 ± 3 and 107 ± 6 mm Hg, respectively. MAP did not change during or after Cinacalcet administration at either dose. We measured both plasma Ca and PTH at the end of the protocol as positive controls. In the vehicle control group, plasma Ca was 1.24 ± 0.02 mmol/L, while in the 1 mg/kg Cinacalcet-treated group and 5 mg/kg Cinacalcet group it was 0.94 ± 0.02 mmol/L (p<0.001) and 0.97 ± 0.02 mmol/L (p<0.001), respectively. Plasma PTH was 114.2 ± 15.1 pg/ml in the vehicle-treated group, 36.0 ± 12.6 in the 1 mg/kg Cinacalcet group (p<0.001) and 26.0 ± 8.3 pg/ml in the 5 mg/kg Cinacalcet-treated group (p<0.001).

II. The effects of acutely-administered Cinacalcet on plasma Ca and PTH over time:

We measured the effects of Cinacalcet on plasma Ca and PTH to test whether Cinacalcet decreased plasma Ca and PTH at all time points in which PRA was measured. Anesthetized rats received the vehicle control, 1 mg/kg Cinacalcet or 5 mg/kg Cinacalcet. The basal plasma Ca in the 5 mg/kg Cinacalcet group was 1.27 ± 0.03 mmol/L. The basal plasma Ca in the vehicle-treated group was 1.20 ± 0.02 mmol/L and did not significantly change and 15, 30 or 60 minutes post-vehicle (1.23 ± 0.05 mmol/L, 1.25 ± 0.04 mmol/L, 1.27 ± 0.03 mmol/L). The basal plasma

Ca in the 1 mg/kg Cinacalcet group was 1.30 ± 0.02 mmol/L. 1 mg/kg Cinacalcet significantly decreased plasma Ca at 15, 30 and 60 minutes post-1 mg/kg Cinacalcet to 1.10 ± 0.08 mmol/L (p<0.05), 1.10 ± 0.02 mmol/L (p<0.05) and 1.07 ± 0.04 mmol/L (p<0.01), respectively. 5 mg/kg Cinacalcet significantly decreased plasma Ca at 15, 30 and 60 minutes post-5 mg/kg Cinacalcet to 1.13 ± 0.01 mmol/L (p<0.01), 1.08 ± 0.02 mmol/L (p<0.01) and 0.98 ± 0.02 mmol/L (p<0.001), respectively.

The basal PTH in the vehicle-treated group was 117.3 ± 23.1 pg/ml and did not significantly change and 15, 30 or 60 minutes post-vehicle (107.9 ± 17.2 pg/ml, 86.7 ± 7.9 pg/ml, 123.1 ± 0.5 pg/ml). The basal PTH in the 1 mg/kg Cinacalcet group was 162.5 ± 9.9 pg/ml. 1 mg/kg Cinacalcet significantly decreased plasma Ca at 15, 30 minutes post bolus to undetectable levels (p<0.001), and 26.9 ± 14.3 pg/ml (p<0.001) at 60 minutes post-bolus. The basal PTH in the 5 mg/kg Cinacalcet group was 118.9 ± 34.1 pg/ml. 5 mg/kg Cinacalcet significantly decreased PTH to undetectable levels (p<0.01) 15 minutes post-bolus, 11.4 ± 11.4 pg/ml (p<0.01) 30 minutes post-bolus, and again to undetectable levels (p<0.05) 60 minutes post-bolus.

III. The effect of acutely-administered Cinacalcet on furosemide-stimulated PRA:

To test whether acute activation of the CaSR with Cinacalcet can inhibit stimulated PRA, we tested the effects of Cinacalcet after administering furosemide to anesthetized rats. The furosemide-stimulated PRA in the vehicle control group was 36.9 ± 4.2 ng Ang I/ml/hr and remained elevated at 15, 30 or 60 minutes post-vehicle (34.4 ± 3.6, 32.5 ± 3.4 ng Ang I/ml/hr and 29.3 ± 3.7 ng Ang I/ml/hr, respectively). Furosemide-stimulated PRA in the Cinacalcet group began at 30.6 ± 2.3 ng Ang I/ml/hr, but was significantly and consistently decreased 15, 30 and 60 minutes post-Cinacalcet to 21.3 ± 2.3 (p<0.001), 22.5 ± 2.2 (p<0.001) and 21.9 ± 1.9 ng Ang I/ml/hr (p<0.001), respectively (Figure 3).

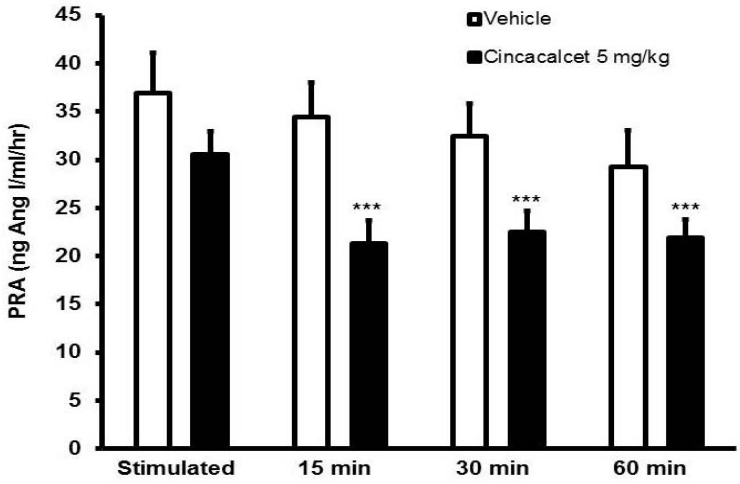


Figure 3: The acute effects of Cinacalcet on Furosemide-stimulated PRA in anesthetized rats. 5 mg/kg Cinacalcet significantly decreased PRA at 15, 30 and 60 minutes post-treatment, respectively. PRA in the vehicle-treated groups did not change at 15, 30 and 60 minutes post-treatment, respectively. *** $p<0.001$ versus stimulated. N=8-9.

MAP after furosemide was 115 ± 4 mm Hg. Cinacalcet slightly, but significantly, increased MAP to 121 ± 4, 15 minutes post-administration ($p<0.05$). MAP returned to normal at 30 minutes (115 ± 4 mm Hg) and decreased at 60 minutes to 107 ± 3 ($p<0.001$).

We also measured the effect of Cinacalcet on plasma Ca and PTH in the presence of furosemide. Plasma Ca was lower in the presence of Cinacalcet plus furosemide (0.92 ± 0.05 mmol/L) versus the vehicle and furosemide group (1.24 ± 0.03 mmol/L, $p<0.001$,), while plasma PTH was lower in the Cinacalcet plus furosemide group (27.1 ± 7.1 pg/ml) compared to the vehicle plus furosemide group (123.7 ± 20.4 pg/ml, $p<0.001$).

IV. The effect of acutely-administered Cinacalcet in PTX rats:

To determine if Cinacalcet inhibits PRA independently of its effects on plasma Ca and PTH, we used PTX rats. Basal PRA in the sham-operated group was 13.5 ± 2.2 ng Ang I/ml/hr and, similar to the PTX group, decreased significantly 15 and 30 minutes post-Cinacalcet to 6.6 ± 0.8 ng Ang I/ml/hr ($p<0.05$) and 6.6 ± 1.5 ng Ang I/ml/hr ($p<0.05$), respectively. PRA at 60 minutes post-Cinacalcet was not significantly different (11.3 ± 2.6 ng Ang I/ml/hr). Basal PRA in the PTX group was 9.3 ± 1.3 ng Ang I/ml/hr and decreased 15 and 30 minutes post-Cinacalcet to 5.2 ± 0.5 ng Ang I/ml/hr ($p<0.05$) and 5.8 ± 0.9 ng Ang I/ml/hr ($p<0.05$), respectively. PRA at 60 minutes post-Cinacalcet was not significantly different (8.6 ± 0.5 ng Ang I/ml/hr).

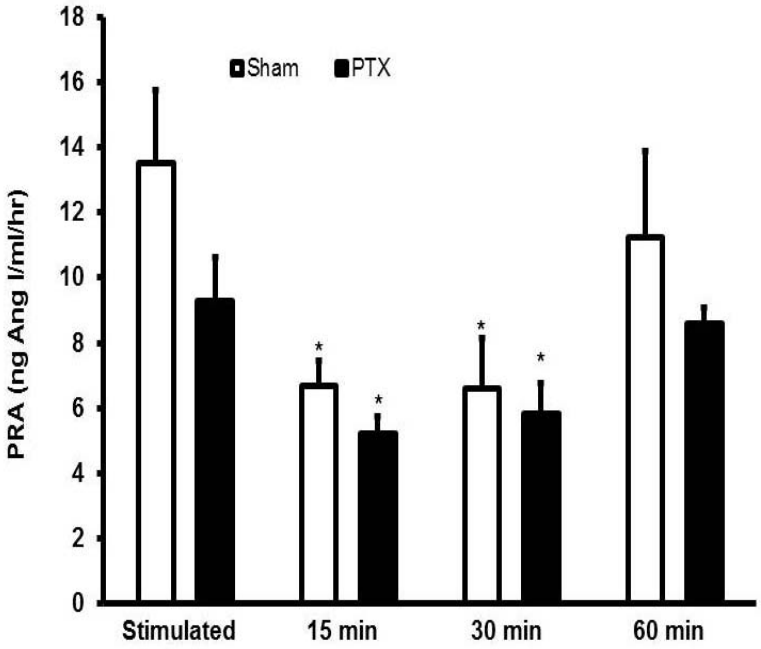


Figure 4: The acute effects of Cinacalcet on unstimulated PRA in anesthetized, para-thyroidectomized (PTX) or sham rats. In the PTX group, 5 mg/kg Cinacalcet significantly decreased PRA at 15 and 30 minutes post-Cinacalcet. However, PRA was not significantly reduced at 60 minutes in the PTX group. In the sham-operated group, 5 mg/kg Cinacalcet significantly decreased PRA at 15 and 30 minutes post-Cinacalcet but not at 60 minutes. No differences between PRA values were found between groups at any time point. *$p<0.05$ vs. basal. N=4-7.

Basal MAP was 108 ± 3 mm Hg in the PTX rats, and 113 ± 4 mm Hg in the sham-operated rats. Cinacalcet did not change the MAP in either group. In the PTX group, basal plasma Ca was 0.85 ± 0.07 mmol/L and did not change in response to Cinacalcet (0.77 ± 0.02 mmol/L). Basal plasma Ca was 1.12 ± 0.08 mmol/L in the sham group, and decreased to 0.80 ± 0.09 after Cinacalcet ($p<0.001$). Basal PTH was undetectable in the PTX group before and after Cinacalcet. Basal PTH in the sham group was 98.3 ± 23.2 pg/ml and decreased to 45.3 ± 17.5 pg/ml after Cinacalcet ($p<0.01$).

V. Chronic protocols with Cinacalcet *via* oral gavage:

We tested whether chronic activation of the CaSR with Cinacalcet could inhibit PRA in conscious rats under unstimulated and stimulated conditions. In the unstimulated groups, the PRA after 7 days of vehicle treatment was 1.6 ± 0.2 ng Ang I/ml/hr and 1.3 ± 0.2 ng Ang I/ml/hr with Cinacalcet treatment. PRA in the groups were not significantly different.

In a second set of rats, PRA was stimulated chronically by Furosemide plus Losartan with or without Cinacalcet over 7 days. The PRA in the stimulated, vehicle-treated rats was 32.1

± 2.5 ng Ang I/ml/hr while the stimulated, Cinacalcet-treated rats was 38.1 ± 4.5 ng Ang I/ml/hr. Thus, the PRA values with Cinacalcet-treatment did not differ from the vehicle-treatment under either basal or stimulated conditions.

Since Cinacalcet, if acting on CaSR, should decrease plasma Ca, we measured the plasma Ca in each group as a positive control. The plasma Ca was lower (0.88 ± 0.03 mmol/L) in the Cinacalcet-treated group compared to 1.02 ± 0.03 mmol/L in the vehicle-treated group under unstimulated conditions ($p<0.01$). Similarly, plasma Ca was lower in the Cinacalcet-treated rats under stimulated conditions compared to vehicle-controls (0.66 ± 0.04 vs. 1.02 ± 0.03, $p<0.001$). As an additional positive control, we measured PTH in the groups with stimulated PRA. As expected, PTH was lower (57.6 ± 6.6 pg/mL) in the Cinacalcet-treated group compared to 97.0 ± 9.8 pg/ml in the control group ($p<0.01$).

DISCUSSION

We have shown that the CaSR coexpresses with renin in the rat renal cortex *in vivo*. We found that activation of the CaSR with Cinacalcet, similar to our *in vitro* results, inhibited basal and stimulated PRA when applied acutely, and that this result was maintained even after the removal of PTH by parathyroidectomy. Thus, our data support our hypothesis that sensitization of the CaSR *in vivo* with Cinacalcet acutely suppresses plasma renin activity.

Renin colocalizes with the CaSR in primary cultures of JG cells from mice.[54] However, to ensure that the colocalization *in vitro* is not artifact, we demonstrated that renin and the CaSR immunofluorescence localize in the same cells in rat renal cortical slices fixed *in vivo*. Our immunofluorescence results provide further evidence of the localization of the CaSR on the JG cells, consistent with previous reports *in vitro*[53,54] and *in vivo*[45].

The ability of Cinacalcet to inhibit PRA is consistent with our previous *in vitro* data demonstrating that the CaSR inhibits renin release from isolated JG cells. The activation of the

CaSR inhibits adenylyl cyclase and decreases cellular cyclic AMP (cAMP) production.[55-56] cAMP is the key second messenger for stimulating renin secretion, and stimuli that decrease cAMP similarly decrease renin.[7] Our studies in isolated JG cells have shown that the acute application of Cinacalcet inhibits both renin release and JG cell cAMP accumulation.[54] The NKCC transport inhibitor, Furosemide, also stimulates renin secretion by increasing JG cell cAMP *via* macula densa-derived PGE_2 production.[50] Our data demonstrate that Cinacalcet inhibits Furosemide-stimulated PRA, which is consistent with our findings that acute activation of the CaSR inhibits renin secretion by directly inhibiting adenylyl cyclase isoform V and JG cell cAMP production[27,53-56], thus blunting the adenylyl cyclase response to classical stimuli for renin secretion.

To show that the Cinacalcet was given at an effective dose, we also measured the responses of plasma Ca and PTH. Calcimimetics are used to treat primary and secondary hyperparathyroidism, as they lower plasma Ca and PTH by increasing the sensitivity of the CaSR to the ambient concentration of extracellular Ca.[31] As expected, at 60 minutes post-Cinacalcet the plasma Ca and PTH were significantly decreased in the Cinacalcet-treated groups compared to vehicle controls in both the presence and absence of furosemide. Similar reductions in plasma Ca and PTH were seen in chronically treated conscious rats. These observations indicate that Cinacalcet increased the sensitivity of the CaSR to extracellular Ca under all of our experimental conditions.

We also tested the effect of Cinacalcet on plasma Ca and PTH at all intermediate time points, to determine whether there was a temporal relationship between the changes in basal PRA, Ca and PTH. Cinacalcet decreased PRA versus basal levels at 15, 30 and 60 minutes post-Cinacalcet. Plasma Ca values decreased progressively at all 3 time points in response to either the 5 mg/kg or 1 mg/kg Cinacalcet bolus. Different still was the response of PTH to Cinacalcet.

PTH was maximally inhibited at 15 minutes, and this inhibition was maintained across all time points.

We also studied the effects of Cinacalcet on PRA in parathyroidectomized rats. The acute infusion of PTH has been reported to increase PRA in dogs,[66] and parathyroidectomies can decrease PRA in patients with hyperparathyroidism and may help normalize blood pressure.[13,25,27] To test if Cinacalcet decreased PRA by inhibiting PTH, we repeated our acute 5 mg/kg Cinacalcet experiments in PTX rats. PTH levels were undetectable in the PTX rats, while Cinacalcet decreased PTH levels in the sham controls. Furthermore, the PTX rats were hypocalcemic compared to the sham controls, indicating the PTX procedure was successful. Cinacalcet decreased PRA just as efficaciously in the PTX rats compared to the shams. Thus, our data suggest Cinacalcet decreases PRA independently of its inhibitory effects on PTH.

High concentrations of Cinacalcet have been reported to acutely increase blood pressure.[23] Thus, we measured MAP in our experiments to ensure that any decreases in PRA we detected were not due to pressor effects working through the renal baroreceptor mechanism. While Cinacalcet did not significantly increase MAP in most of our acute protocols, MAP was slightly and transiently elevated at 15 minutes post- 5 mg/kg Cinacalcet, though only in the furosemide-treated group. Since 5 mg/kg Cinacalcet affected MAP in this protocol, we tested the effect of 1 mg/kg Cinacalcet on PRA. 1 mg/kg Cinacalcet had no effect on MAP at any time point, but also inhibited PRA to the same extent as the 5 mg/kg Cinacalcet dose. There were no other effects on MAP by Cinacalcet in any other protocol. Thus, the effects of Cinacalcet on PRA appear to be independent of its effects on blood pressure.

While Cinacalcet decreased PRA, this was not accompanied by a hypotensive response due to the decrease in PRA. This is not surprising, as renin secretion can undergo acute changes that have no effect on blood pressure or other cardiovascular parameters within the time frame of

our protocols.[5]

While we were able to demonstrate that Cinacalcet could acutely inhibit PRA, Cinacalcet failed to inhibit both basal and stimulated PRA when given chronically over 7 days by oral gavage. Since we observed Ca and PTH decreased with this administration, indicates that Cinacalcet was applied successfully. Cinacalcet increases the sensitivity of the CaSR for extracellular Ca by decreasing the EC_{50} for the CaSR for extracellular Ca. One reason the chronic administration of Cinacalcet may not have decreased PRA was because plasma Ca decreased significantly. Thus, even though the CaSR would be acting at a lower "set point" for extracellular Ca, a lower concentration of extracellular Ca acting on the CaSR should produce lower CaSR activity. In support of this notion, we notice that when we keep extracellular Ca constant in primary cultures of JG cells, Cinacalcet significantly decreases renin release, even after exposure to JG cells for 2 hrs.[55] Additionally, one critique of our work was that using Losartan may be a suboptimal choice from chronically elevating PRA, since the AT-1 receptor acts through G_i subunits to exert many of its G-protein coupled effects in various tissues.[63] Additionally, the CaSR may act through G_i in some tissues.[12] However, this was unlikely to affect our results, as we have shown *in vitro* that inhibiting G_i with pertussis toxin has no effect on the CaSR-mediated inhibition of renin release (unpublished observation).

Another possible reason Cinacalcet only inhibited PRA acutely was due to the presence of barbiturate (Inactin) anesthesia in the acute experiments, but not the chronic ones. Barbiturate anesthesia, including Inactin, increases PRA.[24,34,58] This would account for the elevated basal PRA in our anesthetized rats versus the basal PRA in our unanesthetized rats receiving chronic treatment. While this elevation of PRA by barbiturates may occur *via* adrenergic stimulation[58], some data suggest that this stimulation of renin may be independent of the adrenergic nervous system.[24]

The inverse relationship between extracellular Ca and renin secretion has been referred to as the "calcium paradox".[27,56] Previous work has shown that extracellular Ca inhibits renin release *in vitro* by stimulating the CaSR,[54] which decreases the activity of a calcium-inhibitable isoform of adenylyl cyclase and increases the activity of a calcium-sensitive phosphodiesterase.[53,55,56] Our data suggest that this pathway is not just an isolated *in vitro* phenomenon, and that this pathway described *in vitro* is consistent with the acute response we observe *in vivo*. Importantly, these data support the concept that Ca regulates renin by modulating the enzymes controlling cellular cAMP levels (adenylyl cyclase and phosphodiesterase), and thus, renin secretion.[53-56] However, our data suggest that chronic treatment with Cinacalcet is unlikely to have any long-term effects on PRA, indicating that Cinacalcet will not have any deleterious effects on the PRA levels in patients taking these drugs.

CHAPTER 2: HYPERCALCEMIA REDUCES PLASMA RENIN *VIA* PARATHYROID HORMONE, RENAL INTERSTITIAL CALCIUM, AND THE CALCIUM-SENSING RECEPTOR

ABSTRACT

Acute hypercalcemia inhibits plasma renin activity. How this occurs is unknown. We hypothesized that acute hypercalcemia inhibits plasma renin activity *via* the calcium-sensing receptor due to parathyroid hormone-mediated increases in renal cortical interstitial calcium *via* TRPV5. To test our hypothesis, acute *in vivo* protocols were run in sodium-restricted, anesthetized rats. TRPV5 mRNA was measured with real-time quantitative RT-PCR. Acute hypercalcemia significantly decreased plasma renin activity by 37% from 32.0 ± 3.3 to 20.3 ± 2.6 ng Ang I/ml/hr ($p<0.001$). Acute hypercalcemia also significantly increased renal cortical interstitial calcium by 38% (1.73±0.06 mmol/L) compared to control values (1.25±0.05 mmol/L, $p<0.001$). Plasma renin activity did not decrease in hypercalcemia in the presence of a calcium-sensing receptor antagonist, Ronacaleret (22.8±4.3 vs. 21.6±3.6 ng Ang I/ml/hr). Increasing plasma calcium did not decrease plasma renin activity in parathyroidectomized rats (22.5 ± 2.6 vs. 22.0 ± 3.0 ng Ang I/ml/hr). Parathyroidectomized rats were unable to increase their renal cortical interstitial calcium in response to hypercalcemia (1.01±0.11 mmol/L). Acutely replacing plasma parathyroid hormone levels did not modify the hypercalcemic inhibition of plasma renin activity in parathyroid-intact rats (39.1±10.9 vs. 16.3±3.2 ng Ang I/ml/hr, $p<0.05$). TRPV5 mRNA was decreased by 67% in parathyroidectomized rats ($p<0.001$). Our data suggest that acute hypercalcemia inhibits plasma renin activity *via* the calcium-sensing receptor due to parathyroid hormone-mediated increases in renal cortical interstitial calcium *via* TRPV5.

INTRODUCTION

Renin secretion has a unique relationship with extracellular calcium (Ca) in that increased

extracellular Ca inhibits renin release both *in vitro* and *in vivo*. *In vitro*, increased extracellular Ca inhibits renin release from juxtaglomerular (JG) cells by inhibition of adenylyl cyclase V and stimulation of phosphodiesterase-1C, thus decreasing cAMP, the stimulatory second messenger for renin release.[53-56] *In* vivo, acute hypercalcemia also decreases renin secretion.[40,41,74] However, how hypercalcemia Ca inhibits renin secretion is unknown.

It is possible that hypercalcemia inhibits renin by acting on the calcium-sensing receptor (CaSR). To test this, we will use a CaSR antagonist on hypercalcemia-inhibited PRA. The CaSR is ubiquitously expressed and senses changes in extracellular Ca, transducing these changes into intracellular signaling.[12] *In vivo*, the CaSR is integral for maintaining homeostatic control of plasma Ca. It does so primarily by decreasing parathyroid hormone (PTH) secretion and renal Ca reabsorption in response to hypercalcemia.[12] Recently, we have found that the renin-secreting JG cells express the CaSR, and that stimulating the CaSR decreases renin release *in vitro*[53,54] and plasma renin activity (PRA) *in vivo*.[3] However, due to the ubiquitous expression of the CaSR, it is unknown how increases in plasma Ca could be translated into CaSR-mediated inhibition of renin secretion. The basolateral surface of the JG cells is bathed in the renal cortical interstitium.[7] Thus, in addition to increased plasma Ca, increased renal cortical interstitial Ca could directly stimulate the CaSR to inhibit renin secretion. Increasing plasma Ca has been shown to increase renal cortical interstitial Ca.[48] However, whether increased renal cortical interstitial Ca inhibits renin secretion is unknown.

PTH is an 84 amino-acid peptide released from the chief cells of the parathyroid gland. PTH increases plasma Ca, primarily by stimulating Ca resorption from bone and Ca reabsorption in the kidney. PTH may stimulate Ca reabsorption in the cortical thick ascending limb through paracellular pathways. It is unknown if this increase in Ca reabsorption occurs through the paracellular Ca reabsorption channel, claudin-16. PTH stimulates the renal Ca reabsorption,

predominantly in the distal tubule *via* TRPV5, the distal tubule epithelial Ca transporter.[33] PTH increases the expression of TRPV5 and also increases TRPV5 Ca transport.[19,69] These actions of PTH translate into increases in renal cortical interstitial Ca.[48] As such, hypercalcemia may inhibit renin secretion *via* PTH-mediated increases in renal cortical interstitial Ca through TRPV5.

With these considerations, we hypothesized that acute hypercalcemia inhibits plasma renin activity (PRA) *via* the CaSR due to PTH-mediated increases in renal cortical interstitial Ca *via* TRPV5.

MATERIALS & METHODS

Acute *in vivo* preparation: Male Sprague-Dawley rats were placed on a 0.05% NaCl (Harlan-Teklad, Madison, WI) diet for 10 days prior to *in vivo* experimentation. The low NaCl diet was used to stimulate PRA.[40] Stimulation of basal PRA using dietary sodium restriction was used to amplify the possible inhibition of PRA in response to hypercalcemia. Furthermore, it has been reported that high Ca infusions inhibit elevated PRA more efficaciously than basal PRA.[40,41] Rats were fasted overnight prior to being anesthetized with 125 mg/kg body weight thiobutabarbitol (Inactin, Sigma, St. Louis, MO) and placed on heating pad for the duration of the experiment. Rats were then given a tracheotomy using PE-240 tubing (Clay/Adams Becton Dickinson, Parsipanny, NJ). The femoral vein was catheterized using PE-50 tubing and a maintenance infusion of 10 μl/min of 0.9% NaCl was given. The femoral artery was catheterized with PE-50 tubing attached to a Statham pressure transducer (Viggo-Spectramed, Oxnard, CA), and connected through an iWorx 118 A to D Signal Processor to a computer using iWorx Labscribe 2.065 data acquisition software (iWorx, Dover, NH) for continuous monitoring of mean arterial pressure (MAP). The pressure transducers were calibrated using a digital, mercury-free "traceable" manometer (Fisher Scientific, Pittsburgh, PA). The peritoneal cavity

was opened with a midline incision, and the intestines were wrapped with wet gauze and gently moved under the right abdominal wall to expose the left kidney. A dialysis catheter, consisting of a single 1-cm strand of dialysis tubing (Hemoflow F-8, Fresenius, Waltham, MA) fused to two sections of PE-20 tubing using nail polish (Revlon, New York, NY), was inserted under the renal capsule in the renal cortical interstitium. The dialysis catheter was perfused at a rate of 2 µl/min (see *in situ* dialysis methods below). At the completion of the surgery, the rat received a 1.0 ml bolus of 6% heat-inactivated bovine serum albumin (BSA, Sigma, St. Louis, MO). Any blood withdrawn was replaced with an equal volume bolus of 6% BSA i.v. Rats were allowed to recover from the surgery for 1 hour. Before the commencement of experimental manipulations, blood for basal PRA, PTH and plasma Ca were withdrawn at a volume of 300 µl each. Rats were subjected to various experimental manipulations (listed below) for 90 minutes before blood for PRA, PTH and plasma Ca were withdrawn again. Rats were then euthanized via bilateral pneumothorax and aortic transection. The left kidney was removed for the inspection of any anatomical abnormalities, including ischemia, swelling and hydronephrosis. Rats with visible visceral trauma were excluded from analyses. All procedures were approved by the Henry Ford Health System IACUC committee, which is AAALAC approved, and adhere to the guiding principles in the care and use of experimental animals in accordance with the National Institutes of Health (NIH) guidelines.

***In situ* microdialysis:** Our methods of *in situ* microdialysis are based on the work of R.D. Bukoski.[48] During each experimental period, 3 sequential infusions of salines with different concentrations of Ca were pumped through the dialysis tubing at a rate of 2 µl/min. These salines consisted of 0.9% NaCl with varying concentrations of Ca (0.3 mmol/L, 1.0 mmol/L, 2.2 mmol/L). Each saline was infused through the dialysis tubing over 30 minutes. Only the effluent during the last 20 minutes was collected, in order to allow for the clearing of

the dead space in the tubing.

The concentration of Ca for infusions and their paired effluent collections were measured using a NOVA-8 (NOVA Biomedical, Waltham, MA) electrolyte analyzer. To determine the concentration of Ca in the cortical interstitium, the concentration of Ca in the infused saline and its paired effluent were plotted on a graph, as previously described.[48] The Ca concentration infused was plotted on the X-axis and the paired difference between the effluent Ca concentration and the infusion Ca concentration was plotted on the Y-axis. A line of best fit was generated, and the X-intercept (or point of zero flux) determined the interstitial Ca concentration.[48] Based on preliminary data, we know that our dialysis solutions do no come into complete equilibrium with the cortical interstitium, as a continuous infusion of a constant concentration of Ca through the dialysis tubing does not yield the concentration of Ca in the cortical interstitium. Thus, the entire validity of this technique is based on its ability to measure the flux of Ca that occurs with an infused solution.

Parathyroidectomy (PTX):

Parathyroidectomies were performed as described previously,[3] with the exception that rats were used for *in vivo* protocols 48-72 hrs after the completion of the surgery. PTX rats were placed on the 0.05% NaCl diet for 7-8 days prior to the PTX surgery, identical to rats in the other protocols, and were maintained on 0.05% NaCl diet for the 48-72 hr recovery period prior to any acute experimentation. PTX was confirmed by undetectable plasma PTH levels.

Plasma Renin Activity (PRA):

PRA was analyzed from 300 µl of femoral venous blood. Blood was centrifuged at 16000 x g for 6 min at 4º C and the plasma was aspirated and stored at -20° C until PRA was determined. PRA was analyzed by generation of angiotensin I (Ang I/ hr/min) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) according to the manufacturer's instructions.

Plasma parathyroid hormone (PTH) and ionized Ca:

Measurements of plasma PTH and Ca were performed on femoral venous blood samples. Plasma PTH was measured using a commercial rat PTH immunoassay kit (Alpco Diagnostics, Salem, NH) according to the manufacturer's instructions. Plasma ionized Ca was measured using a NOVA-8 electrolyte analyzer (Nova Biomedical, Waltham, MA).

Real-Time Quantitative RT-PCR:

Real time RT-PCR for TRPV5 was performed by quantitative real-time RT-PCR using a SYBR green method. Custom rat-specific primers from TIB Molbiol (Adelphia, NJ) were used for all PCR reactions. The primer sequences for TRPV5 are; forward: 5`-tgtgagccatttgtaggtcag-3`, reverse: 5`-gaggttgtgggaacttcga-3`. The primer sequences for claudin-16 are; forward: 5`-agtcaatcgcattccagct-3`, reverse:5`-gagtcatcagcgttcaccat-3`. Real time RT-PCR was performed as follows: 1 μg of DNAse-treated total RNA sample was reverse transcribed using random primers and Omniscript reverse transcriptase (Qiagen, Valencia, CA) in a total volume of 20 μl for 1 hr at 37 °C followed by an inactivation step of 95 °C for 5 min. 2 μl of the reverse transcription reaction was then amplified in a Roche version 2.0 lightcycler PCR instrument (Roche, Indianapolis, IN) using SYBR green dye (SA Biosciences, Frederick, MD) and specific primers. Reactions were set up in a final volume of 20 μl, which contained 2 μl of sample, 1 μM each of both the primers and 10 μl of 2x SYBR green PCR mix. After an initial "hot start" at 95°C for 10 min, amplification occurred by denaturation at 95°C for 15s, annealing at 58°C for 45 sec, and extension at 72°C for 1 min for a total of 30-40 cycles. At the end of PCR cycling, melting curve analyses were performed. A relative quantitation method [ΔΔCt][76] was used to evaluate expression of each gene. RT-PCR of GAPDH was used for normalization of all data.

Statistics:

Single changes from baseline to post-treatment were analyzed using a Student's paired *t*-

test. Repeated changes from baseline to post-treatment were analyzed using a One-way, Repeated-Measures ANOVA with a Student-Newman-Keuls test for post-hoc analyses. Intergroup analyses were performed using a One-way ANOVA with a Student-Newman-Keuls test for post-hoc analyses. Values $p < 0.05$ were considered statistically significant. All data are presented as mean ± 1 SEM. For the purpose of simplicity, a single asterisk is used to denote statistical significance in figures, regardless of *p*-value. Actual *p*-values are provided in the text.

***In vivo* protocols**:

Protocol 1: NaCl Control: (*n*=16) Rats were instrumented as described in the acute *in vivo* preparation section. Rats received a control 0.9% NaCl i.v. infusion for the duration of the experiment.

Protocol 2: High-Ca: (*n*=17) Rats received an i.v. infusion of 0.3 mg Ca/kg bodyweight/min (dissolved in 0.9% NaCl, pH 7.4) over the duration of the experimental period. Calcium lactate pentahydrate (Sigma, St. Louis, MO) was the Ca salt used for the infusion to avoid increasing plasma chloride.

Protocol 3a: High-Ca infusion+CaSR inhibition (Ronacaleret): (*n*=8) The calcilytic Ronacaleret was used to inhibit the CaSR. Ronacaleret has a half-life of 4-5 hours *in vivo*.[62] Ronacaleret was generously provided by GlaxoSmithKline, Molecular Discovery Research, Research Triangle Park, N.C. Rats received the same high-Ca infusion as above, but also received the CaSR antagonist Ronacaleret. Ronacaleret was given as a 1 mg/kg bodyweight i.v. bolus in 300 µl 0.9% NaCl every 30 minutes, commencing with the beginning of the high-Ca infusion. As such, each rat received three 1 mg/kg doses of Ronacaleret during the experiment.

Protocol 3b: Ronacaleret control: (n=5) Rats were maintained on 0.05% NaCl chow for 10 days before being anesthetized and instrumented identically to rats from the High-Ca i*n vivo* protocols. Rats were given a bolus of 1 mg/kg i.v. Ronacaleret after the removal of blood for

basal PRA, plasma PTH and Ca measurements. Blood for PRA, PTH and plasma Ca measurements were taken again 30 minutes post-Ronacaleret.

Protocol 4a: High-Ca+parathyroidectomy (PTX): (*n=8*) PTX was performed as described previously.[3] Rats were used 48-72 hours post-PTX for acute protocols. Rats received the same high-Ca infusion as protocol 2, as described above.

Protocol 4b: Extended High-Ca+parathyroidectomy (PTX): (*n=4*) In 4 PTX rats from protocol 4a we continued the high-Ca i.v. infusion for an additional 90 minutes after the removal of blood for the measurement of experimental PRA, Ca and PTH values. During this additional 90 minute i.v. infusion, renal cortical interstitial Ca was measured again. At the end of the additional 90 minute i.v. infusion, blood was withdrawn for a third determination of PRA, plasma Ca and plasma PTH.

Protocol 5: High-Ca +PTH replacement infusion: (n=8) After the withdrawal of blood for the determination of basal PRA, plasma PTH and Ca, rats received a 200 ng/kg i.v. bolus of rat PTH 1-84 (Bachem, Torrance, CA) delivered in a 300 µl bolus of 0.9% saline. During the i.v. high-Ca infusion, rats were concomitantly infused with 20 ng/kg/min of rat PTH 1-84, i.v. This was done to test whether acute changes in PTH were affecting the changes in PRA we saw with our high-Ca infusion, since plasma PTH levels dropped significantly in protocol 2. These rats were parathyroid-intact, not PTX.

Protocol 6: Effect of PTX on TRPV5 mRNA: PTX (n=3) or sham (n=4) rats were anesthetized with Inactin (125mg/kg) 72 hours after recovering from their respective surgeries. The right femoral artery was catheterized and 1 ml of blood was withdrawn for plasma Ca and PTH quantification. The left kidney was exposed *via* a mid-ventral incision, excised and decapsulated before cortical tissue was harvested on an ice-cold Lucite block. Cortical tissue was minced in ice-cold Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) before

being centrifugated at 12,000 g for 10 min at 4º C. The supernatant was collected and snap frozen in liquid nitrogen and stored at -80º C until extraction of RNA.

RESULTS

***In vivo* protocols**:

Protocol 1: NaCl Control: Time controls were run without any change in Ca delivery. Data for protocol 1 are summarized in Figure 5, Figure 6 and Table 1. The control i.v. NaCl infusion had no effect on PRA, plasma Ca, plasma PTH or MAP. Renal cortical interstitial Ca was 1.25±0.05 mmol/L.

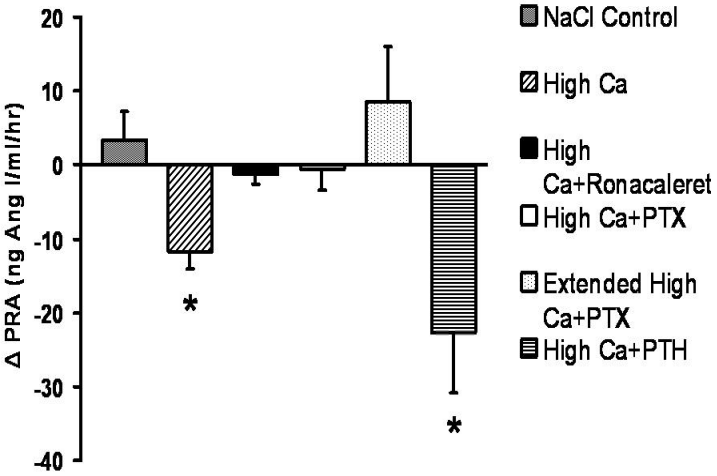


Figure 5: The effects of CaSR, PTX and acute PTH replacement on Ca-mediated PRA inhibition. Increasing plasma Ca decreased PRA by 37%. High plasma Ca did not decrease PRA in the presence of CaSR inhibition (Ronacaleret) or in PTX rats. Increasing plasma Ca decreased PRA by 58% in rats receiving acute PTH replacement. * = $p<0.05$. See text for specific p-values. Data are presented as mean ± 1 SEM. N=4-17.

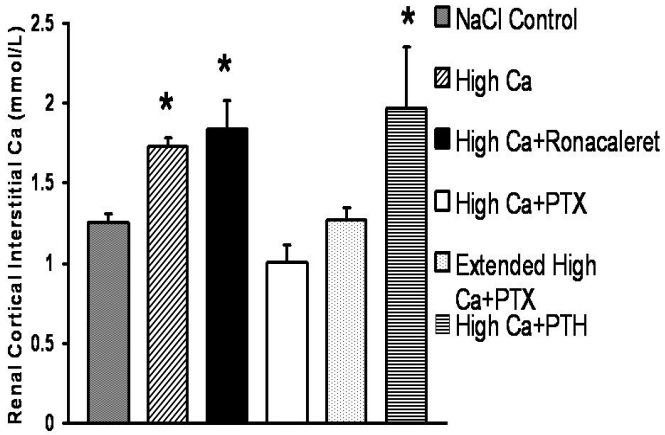


Figure 6: The effects of high plasma Ca on renal cortical interstitial Ca. Increasing plasma Ca increased renal cortical interstitial Ca 38% above i.v. NaCl control values. Renal cortical interstitial Ca was also increased by 47% in the presence of high plasma Ca and CaSR antagonism (Ronacaleret) compared to NaCl controls. Renal cortical interstitial Ca in PTX rats receiving the i.v. high-Ca infusion did not differ from i.v. NaCl controls, even in the presence of the extended i.v. high-Ca infusion. Renal cortical interstitial Ca was elevated 58% over i.v. NaCl control values in rats that received the i.v. high-Ca infusion with PTH replacement. * = $p<0.05$ versus NaCl control. See text for specific p-values. Data are presented as mean ± 1 SEM. N=3-14.

Table 1: Parameters measured in each of the various protocols.

	Basal PRA (ng Ang I/ml/hr)	Experimental PRA (ng Ang I/ml/hr)	Basal Plasma Ca (mmol/L)	Experimental Plasma Ca (mmol/L)	Basal PTH (pg/ml)	Experimental PTH (pg/ml)	Basal MAP (mm Hg)	Experimental MAP (mm Hg)
NaCl I.V. Control	26.7±4.6	30.2±4.9	1.12±0.04	1.18±0.02	104.8±9.8	90.3±8.7	106±3	107±3
High-Ca I.V. Infusion	32.0±3.3	20.3±2.6*	1.19±0.04	1.91±0.07*	171.3±32.9	4.7±4.7*	106±2	117±2*
High-Ca I.V. Infusion+ Ronacaleret	22.8±4.3	21.6±3.6	1.36±0.03	1.77±0.09*	80.4±19.9	34.1±23.6	97 ±2	108±2*
Ronacaleret Control	35.0±11.3	27.2±15.1	1.17±0.04	1.27±0.04*	198.1±5.8	129.2±25.0	104±4	105±5
High-Ca I.V. Infusion+ PTX	22.5±2.6	22.0±3.0	0.62±0.04#	1.50±0.06*	0±0	0±0	104±3	123±4*
Extended High-Ca I.V. Infusion+ PTX	21.9±3.7	30.5±5.7	0.69±0.06#	1.94±0.19*^	0±0	0±0	103±2	122±4*
High-Ca I.V. Infusion+ PTH	39.1±10.9	16.3±3.2*	1.19±0.05	1.81±0.10*	161.4±54.8	542.8±250.9	112±3	128±4*

Rats were maintained on a low-NaCl diet for 10 days prior to being anesthetized and administered the I.V. treatment denoted in the column on the left. * = $p<0.05$ versus paired basal value. # = $p<0.05$ versus basal i.v. high-Ca value. ^ = $p<0.05$ versus i.v. high-Ca infusion + PTX. See text for specific p-values. Data are presented as mean ± 1 SEM.

Protocol 2: High-Ca infusion: The effect of hypercalcemia was tested by increasing the concentration of Ca infused into the circulation. The i.v. high-Ca infusion significantly decreased PRA by 37% ($p<0.001$, Figure 5, Table 1). Plasma Ca significantly increased ($p<0.001$), while plasma PTH significantly decreased ($p<0.001$, Table 1). Furthermore, the i.v. high-Ca infusion significantly increased MAP ($p<0.001$, Table 1). Renal cortical interstitial Ca was significantly elevated compared to the rats treated with the control i.v. infusion (1.73±0.06 mmol/L, $p<0.001$, Figure 6).

Protocol 3a: High-Ca +CaSR inhibition (Ronacaleret): To determine if the response to hypercalcemia was mediated by the CaSR, we used the calcilytic Ronacaleret to block the CaSR. PRA did not change from basal levels in rats that received the i.v. high-Ca infusion and

Ronacaleret (Figure 5 and Table 1). Plasma Ca significantly increased ($p<0.01$). However, plasma PTH did not significantly decrease from basal levels, suggesting that the administration of Ronacaleret was effective at inhibiting the parathyroid gland CaSR (Table 1). As before, MAP increased in the presence of the i.v. high-Ca infusion and Ronacaleret ($p<0.001$, Table 1), and renal cortical interstitial Ca was elevated compared to the NaCl control i.v. infusion (1.84±0.18 mmol/L, $p<0.01$, Figure 6.).

Protocol 3b: Ronacaleret control: To determine any effect of the CaSR blocker alone, we ran time controls without hypercalcemia. Based on preliminary data, 1 mg/kg Ronacaleret had no effect on PRA or PTH 30 min after treatment. Plasma Ca increased 30 minutes after Ronacaleret administration ($p<0.01$, Table 1) indicating the administration of Ronacaleret was successful.

Protocol 4a: High-Ca+parathyroidectomy (PTX): To determine the role of PTH in the hypercalcemia-mediated inhibition of PRA, we repeated our i.v. high-Ca infusion in PTX rats. Basal plasma Ca was decreased in PTX rats ($p<0.001$). When receiving the i.v. high-Ca infusion, PRA did not significantly decrease (Figure 5, Table 1), while plasma Ca significantly increased ($p<0.001$), although the final plasma Ca was less than other groups receiving the high-Ca infusion ($p<0.01$). Furthermore, MAP increased significantly ($p<0.001$). Renal cortical interstitial Ca was not significantly different than the control NaCl rats (1.01±0.11 mmol/L, Figure 6).

Protocol 4b: Extended High-Ca+parathyroidectomy (PTX): Because the i.v. high-Ca infusion did not sufficiently increase plasma Ca in PTX rats (protocol 4a), we continued the infusion for an additional 90 minutes in 4 PTX rats to determine if further increasing plasma Ca could decrease PRA. As summarized in Table 1, plasma Ca increased further ($p<0.05$). PRA remained unaffected by the extended i.v. high-Ca infusion (Figure 5, Table 1). MAP was still

elevated compared to control values (p<0.05). As before, renal cortical interstitial Ca remained low and unchanged, even in the presence of further elevated plasma Ca (1.27±0.07 mmol/L, Figure 6).

Protocol 5: High-Ca +PTH replacement infusion: Acute hypercalcemia decreases PTH, and PTH may stimulate renin secretion.[42,66] As such, we tested whether the decrease in PRA from acute hypercalcemia was due to the acute decrease in plasma PTH. To test this, we infused PTH with the i.v. high-Ca infusion in parathyroid-intact rats. PRA significantly decreased when rats were infused with both i.v. high-Ca and PTH (p<0.05, Figure 5, Table 1). Both plasma Ca (p<0.001) and MAP (p<0.001) increased, while plasma PTH levels did not significantly change from basal levels (Table 1). Renal cortical interstitial Ca was increased compared to rats receiving the i.v. NaCl control infusion (1.98±0.38 mmol/L, p<0.01, Figure 6). Our data exclude the possibility that acute reductions in plasma PTH by the high-Ca infusion are directly causing the decrease in PRA.

Protocol 6: Effect of PTX on TRPV5 mRNA: To determine a potential cause of the impaired renal cortical interstitial Ca response in PTX rats to hypercalcemia, we measured the effects of PTX on the relative quantity mRNA of the distal tubule Ca transporter, TRPV5. Plasma Ca was significantly lower in PTX compared to sham-operated rats (0.78±0.03 mmol/L vs. 1.16±0.03 mmol/L, p<0.001). PTH was undetectable in the PTX group and 114.73±14.6 ng/ml in the sham-operated group (p<0.001), indicating the PTX was successful. As seen in Figure 7, renal cortical TRPV5 mRNA was significantly less in PTX rats compared to sham-operated (0.33±0.08 vs. 1.00±0.05 fold, respectively, p<0.001). Claudin-16 mRNA did not significantly differ between PTX and sham-operated rats (0.73±0.5 vs. 1.04±0.18 fold, respectively).

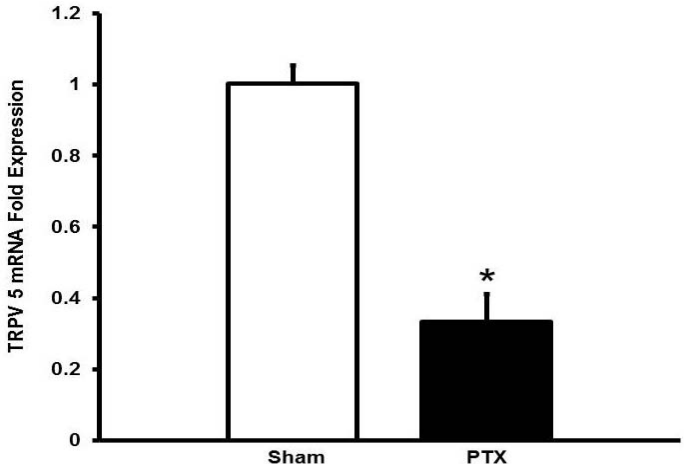


Figure 7: The effect of PTX on renal cortical TRPV5 as measured with real-time quantitative RT-PCR. TRPV5 mRNA was decreased by 67% in PTX rats. * = $p<0.05$ versus sham control. See text for specific *p*-values. Data are presented as mean ± 1 SEM. N=3-4.

DISCUSSION

We have shown that acutely increasing plasma Ca decreases PRA, and that inhibiting the CaSR completely eliminated the hypercalcemia-mediated inhibition of PRA. PTX also completely eliminated the hypercalcemia-mediated decrease in PRA, even when plasma Ca was elevated further for an additional 90 minutes. Noticeably, PTX totally eliminated the change in renal cortical interstitial Ca seen with hypercalcemia. While the i.v. high-Ca infusion decreased plasma PTH, acutely replacing plasma PTH with the i.v. high-Ca infusion did not impair the hypercalcemic inhibition of PRA. TRPV5 mRNA was decreased in PTX rats. These data support the hypothesis that acute hypercalcemia inhibits PRA *via* the CaSR due to PTH-mediated increases in renal cortical interstitial Ca through TRPV5. These data are consistent with the notion of a direct inhibitory effect of Ca on the JG cell.[27,53-56]

PRA is the ability of renin to generate angiotensin I from angiotensinogen endogenous to the plasma and is used as a marker of renin secretion. PRA represents renin synthesized and secreted from the renal cortical JG cells, as PRA from bilaterally-nephrectomized animals decreases to undetectable levels.[38,52] PRA has been shown to be inversely related to the plasma Ca concentration. Similar to our results, acutely increasing the plasma Ca concentration *in vivo* decreases PRA.[40,41,74] However, a mechanism of how this occurred was unknown. It should be

noted that the increase in plasma Ca seen in our protocols, as well as previous experiments examining renin in hypercalcemia, are supraphysiological. [40,41] However, the levels of ionized plasma Ca that we obtain with our i.v. high-Ca infusion (~1.8-1.9 mmol/L) are quantitatively similar to ionized plasma Ca levels take from patients with hypercalcemia of malignancy or hyperparathyroidism (1.72 mmol/L).[75]

We have previously shown that JG cells in rats and mice, both *in vitro* and *in vivo*, contain CaSR.[3,54] The CaSR is a seven-transmembrane domain receptor responsible for homeostatic regulation of plasma Ca.[12] It was first described in the parathyroid gland where it acts by decreasing PTH secretion and increasing urinary Ca excretion in response to hypercalcemia.[12] We have also reported that activation of the CaSR using a calcimimetic *in vivo* decreases PRA.[3] Thus, we hypothesized that the CaSR should also mediate the inhibition of PRA by high plasma Ca. To test this, we used a calcilytic antagonist of the CaSR (Ronacaleret) during hypercalcemia, and found that blocking the CaSR completely eliminated the inhibition of PRA by high plasma Ca (and elevated cortical interstitial Ca). This result confirms that high plasma Ca inhibits PRA by acting on the CaSR.

As a positive control, we also measured the effect of Ronacaleret on the hypercalcemia-mediated inhibition of PTH. The CaSR is also integral in regulating PTH secretion,[12] and calcilytics block both parathyroid gland as well as JG cell CaSR. High plasma Ca negatively feeds back on the parathyroid chief cells to decrease plasma PTH via the CaSR.[12,25] This is illustrated in our results, as we observed that acute hypercalcemia significantly decreased circulating plasma PTH, and this decrease was blunted by CaSR antagonism using Ronacaleret.

PTH is an 84 amino acid peptide secreted from the chief cells of the parathyroid gland. PTH increases plasma Ca by stimulating Ca resorption from bone and also by stimulating renal Ca reabsorption. PTH stimulates renal Ca transport primarily in the distal tubule by increasing

the expression and activation of the epithelial Ca transporter, TRPV5.[19,32,33,69] To test whether acute hypercalcemia inhibits PRA *via* PTH-mediated increases in renal cortical interstitial Ca, we repeated the high-Ca infusion in PTX rats. We found that acute hypercalcemia did not decrease PRA in PTX rats. Furthermore, we found that renal cortical interstitial Ca did not increase with the i.v. high-Ca infusion in PTX rats. However, due to a low basal plasma Ca from the PTX, the high-Ca infusion did not increase plasma Ca to an equivalent level seen in other groups receiving the i.v. high-Ca infusion. To control for this, we extended the i.v. high-Ca infusion in 4 of the PTX rats in an attempt to further increase plasma and renal cortical interstitial Ca. The extended high-Ca infusion increased plasma Ca levels to similar levels seen in other groups with hypercalcemia. However, this extended high-Ca infusion still failed to either decrease PRA or increase renal cortical interstitial Ca in the PTX rats. These data demonstrate that acute hypercalcemia inhibits PRA *via* PTH-mediated increases in renal cortical interstitial Ca.

An important question raised by our experiments is: how could a PTX impair the increase in renal cortical interstitial Ca in response to hypercalcemia? PTH stimulates Ca reabsorption by increasing TRPV5-mediated Ca transport and TRPV5 expression.[19,32,69] As such, we anticipated that our PTX rats could not increase their renal cortical interstitial Ca because of low TRPV5 expression. To test this, we measured renal cortical TRPV5 mRNA in PTX rats and sham controls. As expected, cortical TRPV5 mRNA was significantly decreased in PTX rats compared to sham-operated animals. This indicates that the inability of PTX rats to increase renal cortical interstitial Ca in response to hypercalcemia may be in part due to decreased TRPV5 expression. These data correspond with those of Bindels et al., who found that PTX decreases TRPV5 mRNA and protein, and the addition of PTH to primary cultures of distal tubule cells immediately increases TRPV5 expression and Ca transport.[69] We also

examined the effect of PTX on the claudin-16, the thick ascending limb paracellular Ca reabsorption channel. Renal cortical claudin-16 mRNA was not significantly decreased in PTX rats. This suggests that the impaired renal cortical interstitial response in PTX rats is not due to decreased claudin-16 expression.

We also sought to control for many possible confounding factors in our experiment. PTH has also been reported to stimulate PRA.[42,66] It is possible that this may be due to a direct effect of PTH on the JG cell, as PTH receptors are found in isolated glomeruli with attached vessels.[79] As such, acute hypercalcemia could decrease PRA by acutely decreasing plasma PTH, decreasing the stimulatory effect of PTH on renin secretion. To test if this was the case, we repeated the i.v. high-Ca infusion in parathyroid intact rats with a concomitant i.v. PTH infusion to keep plasma PTH from decreasing. Even when plasma PTH was maintained at physiologic levels in acute hypercalcemia, PRA still decreased. This indicates that acute hypercalcemia does not decrease PRA through acute decreases in PTH.

Changes in blood pressure regulate PRA through the renal baroreceptor mechanism,[65] such that increases in blood pressure decrease PRA. Hypercalcemia increases blood pressure,[68] ostensibly by a direct action on L-type voltage-gated Ca channels on the vascular smooth muscle.[8] As such, we measured the effects of our high-Ca infusion on MAP. The high-Ca infusion increased MAP, as expected. However, it is unlikely that this increase in MAP affected PRA, as it also increased in the Ronacaleret and PTX groups, which experienced no change in PRA. As such, it appears that high plasma Ca does not decrease PRA through increased MAP. Many experimental setups induced renal baroreceptor-mediated changes in PRA *via* instantaneous changes in perfusion pressure. The elevation in MAP we saw with our infusions was a more gradual increase in MAP.[4,71] Thus, we may have avoided renal baroreceptor-mediated changes in PRA due to the rate at which MAP changed. This is consistent with the

previous literature.[40,71] Additionally, when we divided the change in PRA over the change in MAP and compared these values from our High-Ca vs. High-Ca+PTH groups, we saw no difference. As an additional control, we measured the effect of Ronacaleret on basal PRA and found that CaSR blockade had no effect. Thus, Ronacaleret does not stimulate PRA, per se, but inhibits its decline in response to hypercalcemia-mediated CaSR stimulation.

One potential criticism of our work is that our basal PRA values are highly variable. This is due to the low-NaCl diet our rats were placed on, to elevate basal PRA levels and increase the likelihood that we would see an inhibition of PRA by high plasma Ca. This practice is consistent with previous experiments in the literature.[40,41] Additionally, the basal PRA level in the rats receiving the i.v. high-Ca infusion (protocol 2) appears higher than other groups. However, there is no statistical difference between these basal PRA levels, and furthermore, they do not affect our results. When we take rats from protocol 2 with numerically identical baselines to other groups, the i.v. high-Ca infusion still decreases PRA from 21.2±1.6 to 12.3±1.2 ng Ang I/ml/hr (p<0.001, n=9). Our findings are identical for protocol 5; when we take rats with a numerically identical baseline, the i.v. high-Ca infusion with PTH replacement still decreased PRA from 24.2±4.3 to 12.7±2.8 ng Ang I/ml/hr (p<0.05, n=6). As such, apparent, but not actual, baseline PRA differences do not affect our PRA results.

In summary, our data address a 30-year old question in renal physiology: how does acute hypercalcemia inhibit PRA *in vivo*?[40] The answer appears to be that hypercalcemia inhibits PRA by acting on the CaSR. These data are consistent with a direct effect of Ca on the JG cell. The cascade downstream of JG cell CaSR activation, involving inhibition of adenylyl cyclases and stimulation of phosphodiesterases, has already been described in detail.[27,53-56] The chronic presence of PTH is necessary for the inhibition of PRA by hypercalcemia by mediating the increase in interstitial Ca in response to hypercalcemia *via* TRPV5. Our data do not support the

idea that hypercalcemia decreases PRA by acutely decreasing (a renin-stimulating effect of) plasma PTH. All of these data help support the growing body of evidence that the CaSR is an integral component in regulating renin.

CHAPTER 3: THE EFFECTS OF A SUBCAPSULAR CALCIUM INFUSION ON RENAL CORTICAL INTERSTITIAL CALCIUM AND PLASMA RENIN ACTIVITY

ABSTRACT

Hypercalcemia decreases plasma renin activity (PRA) by increasing renal cortical interstitial calcium (Ca). It is unknown whether increased renal cortical interstitial Ca inhibits PRA independently of changes in plasma Ca. We hypothesized that increased renal cortical interstitial Ca can inhibit PRA independently of changes in plasma Ca. Experiments were run acutely in anesthetized rats. Rats received control, intermediate Ca, or high Ca renal subcapsular infusions for the purpose of increasing renal cortical interstitial Ca. Renal cortical interstitial Ca was measured with *in situ* microdialysis. The renal cortical interstitial Ca with the control subcapsular infusion was 1.03±0.05 mmol/L. Renal cortical interstitial Ca did not significantly differ with the intermediate Ca subcapsular infusion (2.57±0.96 mmol/L). However, renal cortical interstitial Ca was significantly elevated in the High Ca subcapsular infusion compared to the control group (3.17±0.58 mmol/L, $p<0.05$). Plasma Ca did not significantly differ between groups. PRA did not significantly change with the control subcapsular infusion. PRA decreased significantly with the intermediate subcapsular infusion, from 49.07±9.35 to 31.54±7.92 ng Ang I/ml/hr ($p<0.05$). PRA did not significantly change in the high Ca subcapsular infusion group. Thus, contrary to our hypothesis, we were unable to consistently inhibit PRA by increasing renal cortical interstitial Ca independently of plasma Ca.

INTRODUCTION

It has been known that plasma renin activity (PRA) is decreased by elevated plasma calcium (Ca).[40,41,74] However, how this occurs is incompletely understood. The renin-secreting juxtaglomerular cells are bordered basolaterally by the renal cortical interstitium *in vivo*. Increasing plasma Ca increases the concentration of Ca in the renal cortical interstitium. Thus,

increasing plasma Ca could inhibit renin secretion by increasing renal cortical interstitial Ca. In support of this, we have previously shown that hypercalcemia both decreases PRA and increases renal cortical interstitial Ca (see Chapter 2). However, this method of increasing renal cortical interstitial Ca required elevated plasma Ca. It is unknown if increasing renal cortical interstitial Ca independently of plasma Ca can decrease renin secretion.

To this end, we hypothesized that increasing renal cortical interstitial Ca independently of plasma Ca would decrease PRA. To test this hypothesis, we employed a subcapsular renal Ca infusion and measured the effects on plasma renin activity (PRA).

MATHERIALS & METHODS

In vivo prep:

Male Sprague-Dawley rats (approximately 200 g, Charles River Laboratory, Wilmington, MA) were placed on a low-salt diet (0.05% NaCl) for 2 weeks. After 2 weeks, they were anesthetized using thiobutabarbital, 125 mg/kg body weight I.P. (Inactin, Sigma, St. Louis, MO) and placed on a heating pad to maintain constant body temperature. A tracheotomy was performed with PE-240 tubing to allow spontaneous breathing of room air. The femoral vein was catheterized with PE-50 tubing for infusion of 10 µl/min 0.9% NaCl. The femoral artery was catheterized with PE-50 tubing attached to a Statham pressure transducer (Viggo-Spectramed, Oxnard, CA), calibrated using a "Traceable" electronic manometer (Fisher Scientific, Pittsburgh, PA), and connected through an iWorx 118 A to D Signal Processor to a computer using iWorx Labscribe 2.065 data acquisition software (iWorx, Dover, NH) for continuous monitoring of mean arterial pressure (MAP). Renal blood flow (RBF) was measured using a 1.5R Transonic Flowprobe (Transonic Systems Inc, Ithaca, NY) placed over the left renal artery. The probe was connected to a T206 small animal blood flow meter (Transonic Systems Inc, Ithaca, NY) and to the iWorx data acquisition software. A microdialysis probe consisting of

a single 1-cm strand of dialysis tubing (Hemoflow F8, Fresenius Medical Care, Lexington, MA), ligated to 2 pieces of PE-20 tubing, was inserted under the renal capsule, with the distal PE tubing protruding from the capsule for the collection of the effluent saline. For the volume expansion and infusion of the renal cortex, 2 separate pieces of PE-20 tubing were inserted under the renal capsule. All perforations of the renal capsule were sealed with Vetbond (3M, St. Paul, MN). Renal venous blood was sampled with a 25-gauge needle, bent to 90 degrees, attached to PE-50 tubing connected to a 1-ml syringe filled with heparinized saline. After surgery, the rats received a supplemental bolus of 1.0 ml of 6% heat-inactivated bovine serum albumin (BSA, Sigma, St. Louis, MO) in normal saline and were allowed to recover for 90 min. All blood samples taken were 300 μL in volume, and were replaced with an equal volume of 6% BSA. When the experiments were completed, the rats were euthanized by pneumothorax and aortic transection, and the kidneys decapsulated, excised, weighed and inspected for anatomical abnormalities.

Plasma Renin Activity (PRA):

PRA was analyzed from 300 μl of femoral venous blood. Blood was centrifuged at 16000 x g for 6 min at 4º C and the plasma was aspirated and stored at -20° C until PRA was determined. PRA was analyzed by generation of angiotensin I (Ang I/ hr/min) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) according to the manufacturer's instructions.

Interstitial dialysis:

To measure interstitial Ca during the subcapsular infusions, 3 different isotonic salines (0.3 mmol/L Ca, 1.0 mmol/L Ca, and 10.0 mmol/L Ca) are perfused through the dialysis tubing at a rate of 2 μl/min for 30 min each. Only the last 20 min of each effluent are collected, as the first 10 minutes allow for the dead space to clear. Once collected, the concentration of Ca in each is measured using an NOVA-8 electrolyte analyzer (Nova Biomedical, Waltham, MA). To

determine the interstitial Ca concentration, a graph is generated with the infused concentrations of Ca on the X-axis, and the difference between the effluent Ca (out) minus the paired infused Ca (in) on the Y-axis. Using these three points, linear regression is used to make a line of best fit. The point at which the line crosses the X-axis (Ca out-Ca in = 0) is the value of the interstitial Ca concentration.

Plasma Ca:

Blood for plasma Ca measurements were taken at the end of the experiment. Plasma Ca was measured with a NOVA-8 electrolyte analyzer (Nova Biomedical, Waltham, MA).

Experimental Groups:

Group 1: Control Ca subcapsular infusion (n=8): After the sampling of basal RSR blood and plasma Ca, we injected a bolus of 250 µl of 1.2 mM Ca (CaCl2) dissolved in 0.9% saline, into the renal cortical interstitium through the PE-20 tubing. After this we infused the renal cortical interstitium with 1.2 mM Ca at 10 µl/min for 15 min. When this was completed, we infused the cortical interstitium with 1.2 mM Ca at 5 µl/min for the remainder of the experiment.

Group 2: Intermediate Ca subcapsular infusion (n=6): This protocol was run identically to Group 1, except 12.5 mM Ca was used as the subcpasular infusate.

Group 3: High Ca subcapsular infusion (n=7): This protocol was run identically to Group 1, except 25 mM Ca was used as the subcapsular infusate.

Statistics:

Repeated changes from baseline to post-treatment were analyzed using a One-way, Repeated-Measures ANOVA with a Student-Newman-Keuls test for post-hoc analyses. Intergroup analyses were performed using a One-way ANOVA with a Student-Newman-Keuls test for post-hoc analyses. Values $p < 0.05$ were considered statistically significant. All data are

presented as mean ± SEM. For the purpose of simplicity, a single asterisk is used to denote statistical significance in figures, regardless of p-value. Actual p-values are provided in the text.

RESULTS

First, we measured the effects of our subcapsular Ca infusions on renal cortical interstitial Ca, using *in situ* microdialysis. This was done to ensure the intermediate and high Ca subcapsular infusions increased renal cortical interstitial Ca. In Figure 8, the renal cortical interstitial Ca was 1.03±0.05 mmol/L with the Control Ca subcapsular infusion. The renal cortical interstitial Ca in the Intermediate Ca subcapsular infusion did not significantly differ from group 1 (2.57±0.96 mmol/L). However, renal cortical interstitial Ca was significantly elevated in the High Ca subcapsular infusion compared to group 1 (3.17±0.58 mmol/L, $p<0.05$).

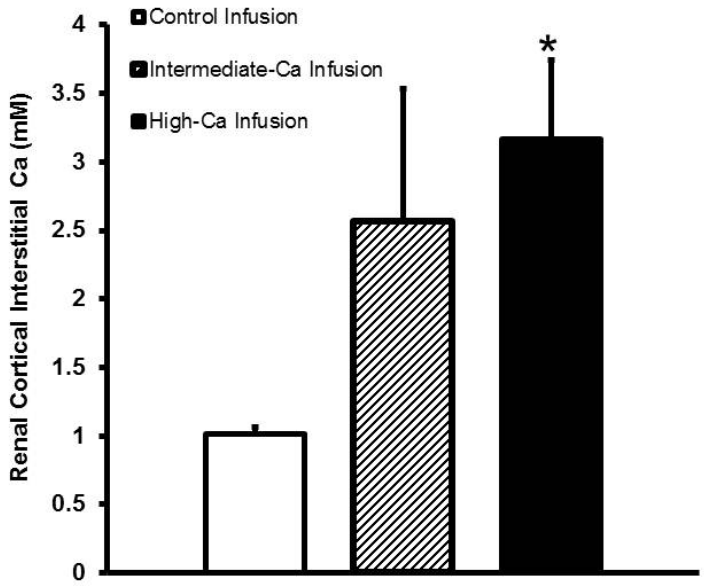


Figure 8: The effects of subcapsular Ca infusions on renal cortical interstitial Ca. The intermediate-Ca subcapsular infusion did not increase renal cortical interstitial Ca compared to control, due to high variability. The high-Ca subcapsular infusion increased renal cortical interstitial Ca approximately 3-fold compared to the control ($p<0.05$). N=6-8.

We also measured our systemic PRA values during the subcapsular infusions (Figure 9, Table 2). PRA did not significantly change during the Control Ca subcapsular infusion. PRA decreased during the Intermediate Ca subcapsular infusion at both 60 and 120 minutes after the start of the subcapsular infusion ($p<0.05$). Systemic PRA did not significantly change during the High Ca subcapsular infusion.

Figure 9: The effects of subcapsular Ca infusions on PRA. The control infusion did not significantly change PRA. PRA decreased with the intermediate-Ca infusion at 60 and 120 minutes ($p<0.05$), coinciding with an increase in MAP seen in table 2. PRA did not change in the high-Ca infusion group. N=6-8.

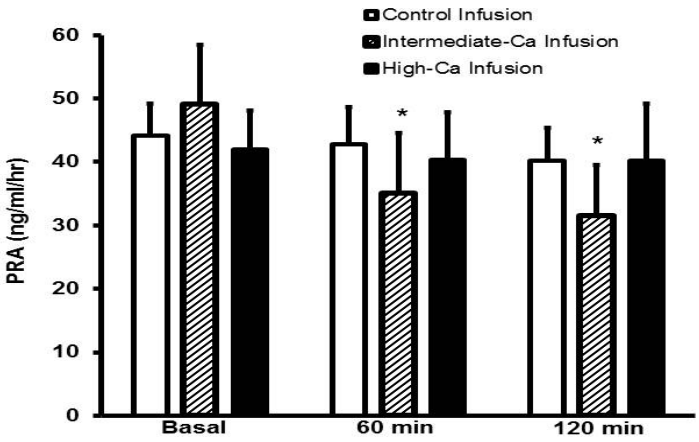


Table 2: The effects of subcapsular-Ca infusions on PRA.

	Basal Systemic PRA (ng Ang I/ml/hr)	60 min Systemic PRA (ng Ang I/ml/hr)	120 min Systemic PRA (ng Ang I/ml/hr)
Control subcapsular infusion	44.09±5.18	42.75±5.86	40.27±5.16
Intermediate- Ca subcapsular infusion	49.07±9.35	35.01±9.54*	31.54±7.92*
High-Ca subcapsular infusion	42.02±6.05	40.35±7.47	40.22±9.05

PRA did not significantly decrease in the control or high-Ca infusion groups. PRA decreased in the intermediate-Ca group at 60 and 120 minutes ($p<0.05$).

To determine if the subcapsular Ca infusions were affecting plasma Ca, we measured plasma Ca at the end of our experiments (Table 3). No significant differences existed between any of the subcapsular infusion groups with respect to plasma Ca levels. Thus, we were able to dissociate changes in renal cortical interstitial Ca from changes in plasma Ca.

Table 3: The effects of subcapsular Ca infusions on plasma Ca, RBF and MAP

	120 min Plasma Ca (mmol/L)	Basal RBF (ml/min/gkw)	60 min RBF (ml/min/gkw)	120 min RBF (ml/min/gkw)	Basal MAP (mm Hg)	60 min MAP (mm Hg)	120 min MAP (mm Hg)
Control subcapsular infusion	1.13±0.05	4.74±0.29	4.76±0.10	4.96±0.093	107±4	108±4	110±4
Intermediate-Ca subcapsular infusion	1.17±0.04	5.95±0.49	5.95±0.69	5.32±0.71	105±3	112±2*	113±1*
High-Ca subcapsular infusion	1.21±0.04	6.01±0.58	5.33±0.63	5.80±0.85	108±3	104±4	106±4

Plasma Ca did not significantly differ between groups. Subcapsular infusions did not affect RBF. MAP increased slightly, but significantly, in the intermediate-Ca subcapsular infusion ($p<0.05$). MAP did not change in the other groups.

We also measured the effects of the subcapsular renal infusions on renal blood flow (RBF) and mean arterial pressure (MAP). As seen in Table 3, RBF did not significantly change in any of the groups receiving a subcapsular infusion. MAP did not significantly change in either the Control or High Ca renal subcapsular infusions. However, MAP did increase in the Intermediate Ca renal subcapsular infusion at both 60 and 120 minutes after the commencement of the subcapsular infusion ($p<0.05$).

DISCUSSION

The aim of our experiments was to decrease PRA by increasing renal cortical interstitial Ca, independently of plasma Ca, using a renal subcapsular infusion. Our results illustrate that our high-Ca subcapsular increased renal cortical interstitial Ca. This increase in interstitial Ca did not affect plasma Ca, indicating we dissociated renal cortical interstitial Ca levels from plasma Ca levels. However, the increase in renal cortical interstitial Ca in the high-Ca group did not decrease PRA, suggesting that increasing renal cortical interstitial Ca independently of plasma Ca does not decrease PRA.

However, we did notice that PRA did decrease in the intermediate-Ca infusion group. But, it is unlikely that the decrease in PRA we saw in this group were due to the intermediate-Ca subcapsular infusion, as we were unable to replicate the decrease in PRA with the high-Ca infusion. As such, we could not consistently decrease PRA by increasing renal cortical interstitial Ca. There were multiple problems with our experimental setup. Infusing solutions into the renal cortical interstitium can increase the cortical hydrostatic pressure and potentially impair the diffusion of Ca across the dialysis membrane. Additionally, we cannot ascertain whether Ca diffused equally across the renal cortical interstitium. Thus, we cannot make any broad conclusions about our experimental results.

CHAPTER 4: THE EFFECTS OF CHANGING RENAL SODIUM CHLORIDE REABSORPTION ON RENAL CORTICAL INTERSTITIAL CALCIUM

ABSTRACT

Elevated thick ascending limb NaCl reabsorption inhibits plasma renin activity (PRA), but it is unknown how this occurs. Elevated thick ascending limb NaCl reabsorption stimulates thick limb calcium (Ca) reabsorption and renal cortical interstitial Ca. Previously, it has been shown that Ca inhibits PRA. Thus, we hypothesize that increased distal NaCl delivery and reabsorption will increase renal cortical interstitial Ca and inhibit PRA. To increase distal NaCl delivery and reabsorption, we performed the following manipulations: Acutely, we used either isotonic or hypertonic, isocalcemic volume expansions in anesthetized rats and measured renal cortical interstitial Ca with *in situ* microdialysis. Chronically, rats consumed a high NaCl diet for 2 weeks prior to renal cortical interstitial Ca measurement. We acutely inhibited thick ascending limb NaCl reabsorption with the loop diuretic furosemide to decrease renal cortical interstitial Ca (and increase PRA) in anesthetized rats. The isotonic volume expansion did not change renal cortical interstitial Ca from 1.44±0.11 mmol/L. Basal renal cortical interstitial Ca before the hypertonic, isocalcemic volume expansion was 1.22±0.08 mmol/L and did not change after the volume expansion (1.20±0.07 mmol/L). Renal cortical interstitial Ca in rats consuming a high-NaCl diet (1.25±0.07 mmol/L) did not significantly differ from rats consuming a normal NaCl diet (1.23±0.08 mmol/L). Inhibiting thick limb NaCl transport with furosemide decreased renal cortical interstitial Ca from 1.20±0.08 mmol/L to 1.05±0.09 mmol/L ($p<0.01$). However, renal cortical interstitial Ca also decreased in our vehicle-controls, from 1.38±0.09 mmol/L to 1.27±0.10 mmol/L ($p<0.05$). Furosemide increased PRA from 12.44±2.05 to 33.77±6.93 ng Ang I/ml/hr ($p<0.05$), while the vehicle-control had no effect. Our data do not support our hypothesis

that increased distal NaCl delivery and reabsorption inhibits PRA by increasing renal cortical interstitial Ca.

INTRODUCTION

Renin is the rate-limiting enzyme of the renin-angiotensin system and is secreted from the juxtaglomerular cells in the afferent arteriole of the kidney. Renin secretion *in vivo* responds to changes in NaCl delivery and reabsorption in the thick ascending limb of the loop of Henle. Specifically, decreased NaCl reabsorption stimulates and increased NaCl reabsorption inhibits renin secretion, respectively.[28,72] While it is known the decreased NaCl reabsorption stimulates renin secretion via increased cyclooxygenase-2 metabolite production at the macula densa,2 it is unknown how increased NaCl delivery inhibits renin secretion.

It has been reported that high-NaCl diets increase renal cortical interstitial Ca.[57] NaCl reabsorption in the thick ascending limb stimulates concomitant calcium (Ca) reabsorption.[9] NaCl reabsorption through the thick ascending limb via the apical NaCl transporter, NKCC2, contributes to the lumen-positive voltage potential in the thick ascending limb.[9] This lumen-positive voltage is the driving force for Ca reabsorption through the paracellular channel, claudin-16.[5] Thus, increased distal NaCl delivery and coupled reabsorption should increase Ca reabsorption and subsequently renal cortical interstitial Ca.

Renin secretion is inhibited by elevated extracellular Ca both *in vivo* and *in vitro*. *In vivo*, the inhibition of renin by high Ca is mediated by increases in renal cortical interstitial Ca that act on the calcium-sensing receptor (CaSR, see Chapter 2). As such, it seemed likely that increased distal NaCl reabsorption would be accompanied by increased Ca reabsorption and therefore, renal cortical interstitial Ca. With these considerations, we hypothesized that increased distal NaCl reabsorption would increase renal cortical interstitial Ca which would inhibit plasma renin

activity (PRA).

To test this hypothesis, we increased distal NaCl delivery with an acute volume expansion or chronically with increased dietary NaCl and measured the effects on renal cortical interstitial Ca. Conversely, we also inhibited NaCl reabsorption with the loop diuretic furosemide, and anticipated that it would both decrease renal cortical interstitial Ca and increase PRA.

MATERIALS AND METHODS

Acute in vivo prep:

Male Sprague-Dawley rats (approximately 200 g, Charles River Laboratory, Wilmington, MA) were anesthetized using thiobutabarbital, 125 mg/kg body weight I.P. (Inactin, Sigma, St. Louis, MO) and placed on a heating pad to maintain constant body temperature. A tracheotomy was performed with PE-240 tubing to allow spontaneous breathing of room air. The femoral vein was catheterized with PE-50 tubing for infusion of 10 μl/min 0.9% NaCl. The femoral artery was catheterized with PE-50 tubing attached to a Statham pressure transducer (Viggo-Spectramed, Oxnard, CA), calibrated using a "Traceable" electronic manometer (Fisher Scientific, Pittsburgh, PA), and connected through an iWorx 118 A to D Signal Processor to a computer using iWorx Labscribe 2.065 data acquisition software (iWorx, Dover, NH) for continuous monitoring of mean arterial pressure (MAP). A microdialysis probe consisting of a single 1-cm strand of dialysis tubing (Hemoflow F8, Fresenius Medical Care, Lexington, MA), ligated to 2 pieces of PE-20 tubing, was inserted under the renal capsule, with the distal PE tubing protruding from the capsule for the collection of the effluent saline. After surgery, the rats received a supplemental bolus of 1.0 ml of 6% heat-inactivated bovine serum albumin (BSA, Sigma, St. Louis, MO) in normal saline and were allowed to recover for 90 min. All blood

samples taken were 300 μL in volume, and were replaced with an equal volume of 6% BSA. Urine was collected by voiding the bladder with a 25-gauge needle attached to a 1 ml syringe. When the experiments were completed, the rats were euthanized by pneumothorax and aortic transection, and the kidneys decapsulated, excised, weighed and inspected for anatomical abnormalities.

Interstitial dialysis:

To measure interstitial Ca during the subcapsular infusions, 3 different isotonic salines (0.3 mmol/L Ca, 1.0 mmol/L Ca, and 2.0 mmol/L Ca) are perfused through at a rate of 2 μl/min for 30 min each through the dialysis tubing. Only the last 20 min of each effluent are collected, as the first 10 minutes allow for the dead space to clear. Once collected, the concentration of Ca in each is measured using an NOVA-8 electrolyte analyzer (Nova Biomedical, Waltham, MA). To determine the interstitial Ca concentration, a graph is generated with the infused concentrations of Ca on the X-axis, and the difference between the effluent Ca (out) minus the paired infused Ca (in) on the Y-axis. Using these three points, linear regression is used to make a line of best fit. The point at which the line crosses the X-axis (Ca out-Ca in = 0) is the value of the interstitial Ca concentration.

Plasma Renin Activity (PRA):

PRA was analyzed from 300 μl of femoral venous blood. Blood was centrifuged at 16000 x g for 6 min at 4º C and the plasma was aspirated and stored at -20° C until PRA was determined. PRA was analyzed by generation of angiotensin I (Ang I/ hr/min) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) according to the manufacturer's instructions.

Statistics:

Single changes from baseline to post-treatment were analyzed using a Student's paired *t*-

test. Intergroup analyses were performed using a Student's *t*-test. Values $p < 0.05$ were considered statistically significant. All data are presented as mean ± 1 SEM. For the purpose of simplicity, a single asterisk is used to denote statistical significance in figures, regardless of *p*-value. Actual *p*-values are provided in the text.

***Experimental Groups*:**

Set 1: Acute Volume Expansion

Group 1: isotonic saline volume expansion (n=3): Rats were instrumented as described above. A basal renal cortical interstitial Ca measurement was made prior to initiating a 3% bodyweight, 0.9% NaCl volume expansion *via* the femoral vein catheter, delivered over 30 minutes. Immediately after the volume expansion, renal cortical interstitial Ca was measured again.

Group 2: hypertonic, isocalcemic saline volume expansion (n=12): This protocol was run identically to group 1, with the exception that the rats received a 1.5% bodyweight, 1.8% NaCl volume expansion containing 1.2 mmol/L Ca.

Set 2: Chronic high NaCl diet.

Group 3: Normal NaCl diet (n=5): Rats were maintained on a normal NaCl diet (0.49%) for 2 weeks prior to being instrumented for renal cortical interstitial Ca measurements. 3 Rats were also placed in metabolic caging for the measurement of 24-hour urinary volume at various time points prior to renal cortical interstitial Ca measurement

Group 4: High NaCl diet (n=4): Rats were maintained on a high NaCl diet (3.2%) for 2 weeks prior to being instrumented for renal cortical interstitial Ca measurements. 3 Rats were also placed in metabolic caging for the measurement of 24-hour urinary volume at various time points prior to renal cortical Ca measurement.

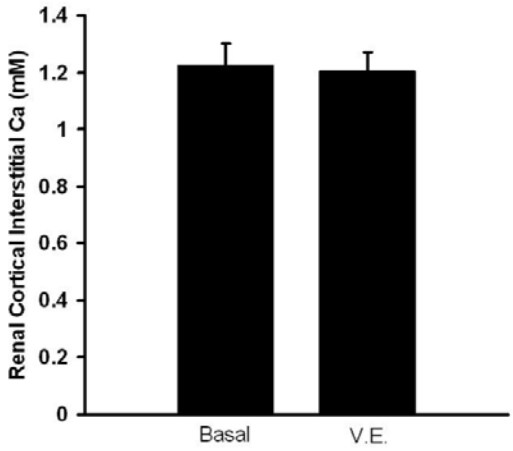


Figure 10: The effect of an acute, hypertonic, isocalcemic volume expansion (V.E.) on renal cortical interstitial Ca. The volume expansion did not significantly change renal cortical interstitial Ca. N=12.

Set 3: Inhibition of thick ascending limb NaCl reabsorption with furosemide.

Group 5: control (n=4): Rats were instrumented as described in the *in vivo* prep section. Basal PRA, renal cortical interstitial Ca and urinary volume measurements were taken before giving a 500 µl bolus of 0.9% NaCl, i.v. Renal cortical interstitial Ca was then measured after the i.v. bolus and PRA and urinary volume were measured at the end of the experiment.

Group 6: furosemide (n=8): Experiments were run identically to group 3, except that rats received 5 mg/kg furosemide delivered in a 500 µl bolus of 0.9% NaCl, i.v.

RESULTS

Set 1: Acute Volume Expansion

Group 1: isotonic saline volume expansion: Basal renal cortical interstitial Ca was 1.44±0.11 mmol/L. The 3% bodyweight volume expansion did not significantly change renal cortical interstitial Ca (1.18±0.03 mmol/L).

Group 2: hypertonic, isocalcemic saline volume expansion: Basal renal cortical interstitial Ca was 1.22±0.08 mmol/L. As seen in Figure 10, renal cortical interstitial Ca did not significantly differ after the hypertonic, isocalcemic volume expansion (1.20±0.07 mmol/L).

Set 2: Chronic high NaCl diet.

Group 3: Normal NaCl diet: Renal cortical interstitial Ca was 1.23±0.08 mmol/L after 2

weeks on a normal NaCl diet. The rats in this group produced 10.0±2.9 ml of urine in 24 hrs, and rats in this group excreted 0.75±0.33 mmoles Na /24 hrs.

Group 4: High NaCl diet: Renal cortical interstitial Ca in the high dietary NaCl group was 1.25±0.07 mmol/L, and as seen in Figure 11, this did not differ significantly from the rats on the normal NaCl diet (group 3). The rats on the high NaCl diet urinated significantly more than rats on the normal NaCl diet (73.3±9.7 ml of urine in 24 hrs, $p<0.01$), and excreted more Na (17.7±2.6 mmoles Na/24 hrs, $p<0.01$).

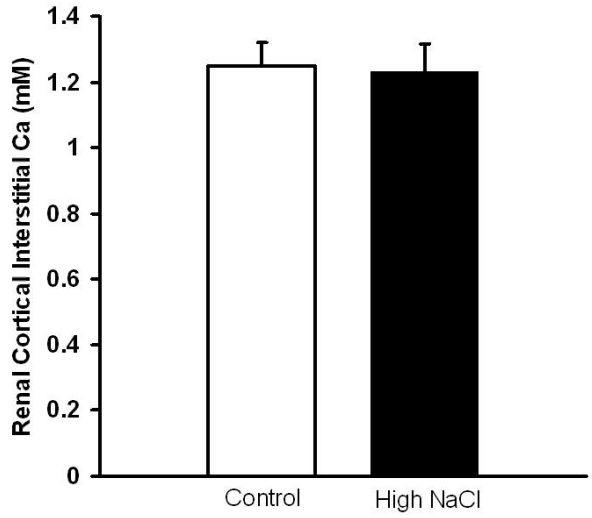
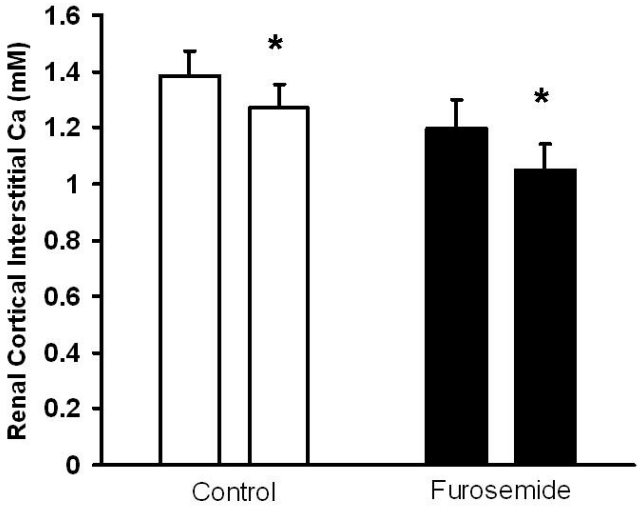


Figure 11: The effect a chronic, high NaCl diet (3.2%) on renal cortical interstitial Ca. Rats were maintained on control or high NaCl diets for 2 weeks prior to renal cortical interstitial Ca measurement. Renal cortical interstitial Ca in the high NaCl diet group did not significantly differ from the control group. N=4-5.

Set 3: Inhibition of thick ascending limb NaCl reabsorption with furosemide.

Group 5: control: Basal renal cortical interstitial Ca was 1.38±0.09 mmol/L and decreased slightly but significantly with the control bolus to 1.27±0.10 mmol/L (Figure 12, $p<0.05$). Basal PRA was 18.86±2.88 ng Ang I/ml/hr and did not change significantly with the saline control bolus (14.57±5.00 ng Ang I/ml/hr). Urinary volume prior to the bolus (0.64±0.09 ml) did not significantly differ from that after the control bolus (0.84±0.21 ml).

Figure 12: The effect of furosemide on renal cortical interstitial Ca. Renal cortical interstitial Ca was measured over 90 minutes, both before and immediately after an i.v. control bolus or a bolus of furosemide. Basal interstitial Ca is the left bar in each group, while the experimental value is on the right. The control i.v. bolus slightly, but significantly, decreased renal cortical interstitial Ca. Furosemide also significantly decreased renal cortical interstitial Ca. * = p<0.05 vs. basal. N=4-8.



Group 6: furosemide: Basal renal cortical interstitial Ca was 1.20±0.08 mmol/L and decreased significantly with furosemide to 1.05±0.09 mmol/L (Figure 12, $p<0.01$). Furosemide significantly increased PRA from 12.44±2.05 to 33.77±6.93 ng Ang I/ml/hr (Figure 13, $p<0.05$). Furosemide increased urinary volume from 0.67±0.16 to 5.60±0.96 ml during the collection period ($p<0.05$).

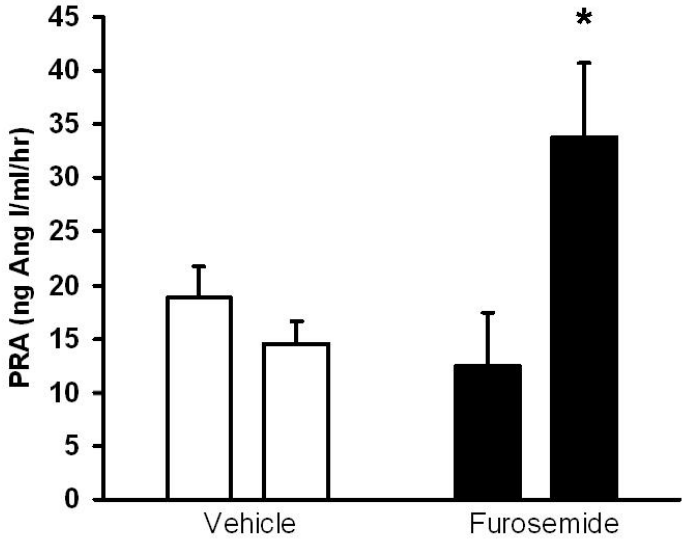


Figure 13: The effect of furosemide on PRA. PRA was measured at the end of the basal and experimental renal cortical interstitial Ca measurement periods. The basal measurement is the left bar of each group, and the experimental value is the right bar in each group. The control bolus had no effect on PRA. Furosemide significantly increased PRA, consistent with previous reports.[8,9] * = $p<0.05$ vs. basal. N=4-8.

DISCUSSION

Based on previous reports, we anticipated that increasing distal NaCl delivery and reabsorption should increase renal cortical interstitial Ca. We hypothesized that increased distal

NaCl reabsorption would increase renal cortical interstitial Ca which would inhibit PRA. However, neither the isotonic nor hypertonic, isocalcemic volume expansions increased renal cortical interstitial Ca. Additionally, chronically increasing distal NaCl delivery and reabsorption with a high-NaCl diet did not increase renal cortical interstitial Ca. Conversely, when inhibiting distal NaCl reabsorption with the loop diuretic furosemide, we saw small but significant decreases in renal cortical interstitial Ca. However, we saw the same response in our vehicle controls. Contrary to our hypothesis, our data show that both increased and decreased NaCl delivery and reabsorption in the thick ascending limb were unable to significantly change renal cortical interstitial Ca.

The most likely explanation for the discrepancy between our proposed and actual results relates to the changes in volume associated with changes in NaCl delivery. Volume expansion inhibits Ca reabsorption.[1,59] Therefore, in the acute volume expansion protocols, any stimulatory effect of NaCl reabsorption on renal cortical interstitial Ca may have been negated by the concomitant volume delivered. A similar effect also could account for the inability of the high-NaCl diet to increase renal cortical interstitial Ca. The rats on the high-NaCl diet were likely volume expanded, as evidenced by their sevenfold higher urinary flow rate.

Our results with the high NaCl diet contrast with those from Bukoski's group, who showed a significant increase in renal cortical interstitial Ca in response to high dietary NaCl.[57] There are differences in the two studies' protocols. We employed a 3.2% NaCl diet, which has 6.5-fold greater NaCl than the normal NaCl diet. Na excretion increased 10-fold on this diet, suggesting the high-NaCl diet was being ingested. However, in the Bukoski study, the high-NaCl diet contained twentyfold more NaCl than controls, but they only excreted twice as much NaCl. Bodyweights were not reported. This suggests that the rats on the high NaCl diet in their

study were not consuming their high-NaCl diet, and the stress of reduced caloric intake may have affected renal cortical interstitial Ca.

Finally, we found that inhibiting thick ascending limb NaCl reabsorption with furosemide slightly but significantly decreased renal cortical interstitial Ca. However, in these studies, our vehicle controls demonstrated a similar effect, suggesting that the decrease in renal cortical interstitial Ca was not furosemide-specific. PRA increased with furosemide, indicating that its administration was successful. Furosemide is a well-characterized stimulus for renin secretion.[4,70] The inability of furosemide to decrease renal cortical interstitial Ca was once again likely due to a concomitant effect on circulating volume. As a potent diuretic, furosemide significantly increased the urinary flow rate, possibly causing acute volume depletion. It is well known that volume contraction caused by diuretics can increase proximal tubule Ca reabsorption,[51] which would negate any inhibitory effect of furosemide on Ca reabsorption. Thus, while furosemide stimulated renin secretion, its pathway appears to be independent of decreased renal cortical interstitial Ca.

In conclusion, our data demonstrate that acute and chronic changes in distal NaCl delivery and reabsorption do not affect renal cortical interstitial Ca, possibly due to the confounding effects of volume associated with the NaCl loads delivered. This raises the question, how does high NaCl inhibit renin? A likely answer has been provided the work of Schnermann et al., suggesting that the inhibition of PRA by high-NaCl is mediated by adenosine release from the macula densa.[39] Adenosine is a metabolic by-product of NaCl reabsorption in the thick limb and macula densa, and has been shown to inhibit renin secretion.[39,49] Taken together, these data suggest that, contrary to our hypothesis, increased NaCl reabsorption in the thick ascending limb of the loop of Henle does not affect secretion *via* changes in renal cortical

interstitial Ca.

PERSPECTIVES:

In conclusion, the sum of our data show that the CaSR is expressed in the renin-containing JG cells *in vivo*, and the stimulating the CaSR acutely inhibits PRA. We have also shown that hypercalcemia inhibits PRA via the CaSR and PTH-mediated increases in renal cortical interstitial Ca via TRPV5. Directly increasing renal cortical interstitial Ca independently of the plasma does not consistently affect PRA. Lastly, changes in PRA caused by changes in renal NaCl delivery and reabsorption are not due to renal cortical interstitial Ca. Thus, our data support our hypothesis that hypercalcemia inhibits PRA via PTH-mediated increases in renal cortical interstitial Ca that act on the CaSR.

Our *in vivo* data, and previous *in vitro* data, provide a novel, unified hypothesis for how acute hypercalcemia inhibits renin release from the JG cells. PTH is necessary for the TRPV5-mediated increase in renal cortical interstitial Ca in response to hypercalcemia. Increased renal cortical interstitial Ca stimulates the JG-cell expressed CaSR. This increases JG cell intracellular Ca, which decreases cellular cAMP via decreased adenylate cyclase activity and increased phosphodiesterase activity. Since cAMP is the major stimulatory second messenger for renin release, this Ca-mediated decrease in cAMP retards renin secretion. The data from this thesis are crucial, because they demonstrate that high plasma Ca inhibits renin both *in vitro* and *in vivo* by similar mechanisms.

The last major question to address with our research is, how does this relate to pathophysiology? While we have used high levels of hypercalcemia to amplify our results, it is likely that more subtle interactions between Ca reabsorption, interstitial Ca concentrations, and cAMP-mediated renin secretion take place constantly at lesser changes levels of Ca. While

chronic hypercalcemia from paraneoplastic syndromes or tumor metastases is more likely to be seen in the clinic, there are many such instances in which patients may be subject to acute hypercalcemia as well. Transient hypercalcemia can occur in acute renal failure, hemodialysis, rhabdomyolysis, thyrotoxicosis, milk-alkali syndrome, or Williams syndrome. Interestingly, it has been noted that many patients with rhabdomyolysis develop both hypocalcemia, as well as elevated PRA levels during acute renal failure, consistent with the effects of Ca on renin that we have described. Whether these changes in Ca are what initiate these changes in PRA remains unknown. The level of hypercalcemia we achieve in our experiments is similar to that seen in patient populations, and as such, it likely provides an accurate model for many of the vascular issues these patients face.

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ABSTRACT

MODULATION OF RENIN SECRETION BY RENAL CORTICAL INTERSTITIAL CALCIUM

by

DOUGLAS K. ATCHISON

May 2012

Advisor: Dr. William Beierwaltes

Major: Physiology

Degree: Doctor of Philosophy

Renin is secreted from the juxtaglomerular (JG) cells of the afferent arteriole of the kidney, and is the rate-limiting enzyme of the renin-angiotensin system. Renin is quantified *in vivo* as plasma renin activity (PRA). Acutely elevating plasma calcium decreases PRA, but the mechanisms by which this occurs is unknown. The overall goal of our study was to determine how elevated plasma calcium decreases PRA. The calcium-sensing receptor (CaSR) is a ubiquitously expressed receptor that translates changes in plasma calcium into changes in intracellular signaling. JG cells are basolaterally bordered by the renal cortical interstitium, and parathyroid hormone (PTH) positively regulates the concentration of calcium in the renal interstitium. Thus, we hypothesized that hypercalcemia inhibits PRA *via* PTH-mediated increases in renal cortical interstitial Ca that act on the CaSR triggering Ca-mediated inhibition of renin secretion. We demonstrated that the CaSR is expressed in JG cells *in vivo*. Additionally, we demonstrated that acutely stimulating the CaSR with pharmacological agonists decrease basal and stimulated PRA in anesthetized rats in parathyroid-intact and parathyroidectomized (PTX) rats. We demonstrated that acute hypercalcemia decreased PRA, and that this inhibition of PRA is blocked by pharmacological CaSR antagonists and by PTX.

Acute supplementation of PTH did not affect the inhibition of PRA by high calcium. Acute hypercalcemia increased renal cortical interstitial calcium, and this effect was blocked by PTX. This was likely due to decreased expression of the PTH-sensitive distal tubule calcium transporter TRPV5. Lastly, we demonstrated that increasing renal sodium chloride delivery failed to increase renal cortical interstitial calcium, and that inhibiting thick ascending limb sodium chloride reabsorption with Furosemide increased PRA without effecting major changes in renal cortical interstitial calcium. Our data support the notion that hypercalcemia inhibits PRA *via* PTH-mediated increases in renal cortical interstitial Ca that act on the CaSR.

AUTOBIOGRAPHICAL STATEMENT

DOUGLAS K. ATCHISON

Education

M.D./Ph.D.: Wayne State University, Detroit, MI (8/07-Present) Degree: Physiology
B.S. with High Honors: Michigan State University, Lansing, MI,. (2001-2005)

Experience

1. Clinical Volunteer (09/03-12/03) - Cristo Rey Community Clinic, (Peter Cooke, M.D.)
2. Research Technician (08/05-08/06) Michigan State University (Gregory Fink, Ph.D.)

Awards

- Dean's List: Fall Semester 2001, Fall Semester 2002
- Dean's List with 4.0 GPA: Spring 2002, Spring 2003, Fall 2004
- 2006 Undergraduate Employee of the Year Runner Up, Michigan State University
- WSU Board of Governors Scholarship, 2006-2008.

Peer reviewed publications

1. **Atchison DK**, Ortiz-Capisano MC, Beierwaltes WH. Acute activation of the calcium-sensing receptor inhibits plasma renin activity *in vivo*. *Am J Physiol Regul Integr Comp Physiol*. 2010 Oct;299(4):R1020-6.
2. **Atchison DK**, Beierwaltes WH. Acute hypercalcemia inhibits plasma renin activity via the calcium-sensing receptor and parathyroid hormone. *Hypertension*. 2011 Oct;58(4):604-10.
3. **Atchison DK**, Westrick E, Szandzik D, & Beierwaltes WH. Parathyroid Hormone-Related Protein Stimulates Plasma Renin Activity via its Anorexic Effects on Sodium Chloride Intake. *Am J Physiol Endo Metab*. In submission.

Abstracts

1. **Atchison D.K**., Harding P., Ortiz-Capisano C., Beierwaltes W.H. Calcium-inhibitable isoforms of adenylyl cyclase are not expressed in the rat parathyroid EB April 2007.
2. **Atchison D.K.**, Beierwaltes W.H. Calcium-sensing receptor activation decreases plasma renin activity *in vivo*. EB April 2009. New Orleans, LA.
3. **Atchison D.K**., Beierwaltes W.H. Inhibition of NaCl Reabsorption with Furosemide Decreases Renal Cortical Interstitial Calcium While Stimulating Plasma Renin Activity. Abstract published at the Council for High Blood Pressure National Meeting, Sept 2009. Chicago, IL. Abstract # 519.
4. **Atchison D.K**., Woo R.E., Beierwaltes W.H. Parathyroid Hormone Stimulates Renin Release From Isolated Juxtaglomerular Cells *Via* Parathyroid Hormone Receptor-1. Abstract published at the Council for High Blood Pressure National Meeting, October 2010. Washington, D.C.
5. **Atchison DK**, Beierwaltes WH. Elevated renal cortical interstitial calcium acutely stimulates renin secretion in anesthetized Sprague-Dawley rats. EB, April 2011. Washington, D.C. Abstract #542.
6. **Atchison DK**, Harding P, Beierwaltes WH. Parathyroid Hormone Related Protein dissociates increased cAMP from Renin Secretion in Mouse Juxtaglomerular Cells. Amer. Soc. Nephrology Renal Week Meeting, Nov 2011. Abstract # 24782
7. **Atchison DK**, Westrick E, Szandzik D, and Beierwaltes WH. Parathyroid Hormone-Related Protein Stimulates Plasma Renin Activity via its Anorexic Effects on Sodium Chloride Intake. EB 2012

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