Calcitonin-stimulated renal $Ca^{2+}$ reabsorption occurs independently of TRPV5

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Abstract

Background. Calcitonin (CT) is known to affect renal $Ca^{2+}$ handling. However, it remains unclear how CT affects $Ca^{2+}$ transport in the distal convolutions. The aim of this study was to investigate the contribution of the renal epithelial $Ca^{2+}$ channel, transient receptor potential vanilloid 5 (TRPV5), to renal $Ca^{2+}$ handling in response to CT.

Methods. C57BL/6 mice received a single overnight (16 hr) injection of CT. In addition, TRPV5 knockout (TRPV5$^{−/−}$) mice and their wild type (TRPV5$^{+/+}$) controls, received three bolus injections of CT over a 40-hr study period. All experimental groups were placed in metabolic cages.

Results. C57BL/6 mice received a single bolus injection of CT, which significantly reduced the urinary $Ca^{2+}$ excretion. In addition, urinary Na$^+$ and K$^+$ excretion also decreased after CT administration. No apparent changes in renal expression of TRPV5, calbindin-D28K (CaBP28K) or TRPV6 could be detected between CT- and vehicle-treated mice. To evaluate whether TRPV5 activity is needed for the CT-induced increase in $Ca^{2+}$ reabsorption, mice with genetic ablation of TRPV5 (TRPV5$^{−/−}$) were employed. TRPV5$^{−/−}$ mice as well as their wild-type (TRPV5$^{+/+}$) controls received three bolus injections of CT over a 40-hr study period. Overnight (16 hrs) as well as the subsequent 24-hr urine was collected. No apparent changes in renal expression of TRPV5 and CaBP28K after three bolus injections of CT. The subsequent 24-hr urinary excretion of $Ca^{2+}$ which was collected after the third bolus injection showed no effect of CT on renal $Ca^{2+}$ handling in either mice group. Accordingly, CT did not alter the intrarenal protein abundance of TRPV5 and CaBP28K after three bolus injections of CT.

Conclusion. CT augments the renal reabsorptive capacity for $Ca^{2+}$. This increase is likely to occur independently of TRPV5.

Keywords: calbindin; calcitonin; calcium; distal convoluted tubule; TRPV5

References


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Introduction

Disturbances in the systemic Ca\(^{2+}\) concentration often result in instability of the neurological and cardiac systems (e.g. hypercalcemia causes fatigue, nausea and abnormal heart rhythms [1]). Thus, maintenance of serum Ca\(^{2+}\) concentration plays an important role in stabilizing these physiological processes. Several hormones have been known to affect the systemic Ca\(^{2+}\) concentration. Calcitonin (CT) is a 32 amino acid peptide synthesized by post-translational processing in the C cells of the thyroid gland in mammals [2]. CT acts primarily by inhibiting osteoclast-mediated bone resorption and is secreted in response to increases in systemic Ca\(^{2+}\) concentration [2]. Historically, CT has been extensively used in treating hypercalcemia, osteoporosis and Paget’s disease [2]. Throughout the kidney and elsewhere, CT acts primarily by activating the adenylate cyclase pathway [3,4].

In the kidney, vectorial NaCl transport within the thick ascending limb (TAL) drives paracellular transport of Ca\(^{2+}\). Administration of CT to rats increases the renal reabsorption of Ca\(^{2+}\), which is in part achieved by increasing the reabsorption of Ca\(^{2+}\) in the TAL. Here, application of CT also increases vectorial NaCl transport [5–7]. Active transcellular Ca\(^{2+}\) transport is restricted to the distal convolutions. Here, luminal Ca\(^{2+}\) enters across the apical membrane via the epithelial Ca\(^{2+}\) channel, transient receptor potential vanilloid 5 (TRPV5) [8,9]. Studies in rabbit suggest that CT exerts its effect on Ca\(^{2+}\) reabsorption only in the distal convoluted tubule (DCT), while no stimulation occurs in the connecting tubule (CNT) [10]. In addition, CT fails to stimulate cyclic adenosine monophosphate (cAMP) accumulation in the CNT of rabbits [11]. Our group has also been unable to show stimulatory effects of CT on cAMP production in rabbit primary CNT cultures [12]. In the rabbit, TRPV5 localizes primarily to the CNT where CT does not affect Ca\(^{2+}\) reabsorption [8,10]. However, in mouse, TRPV5 is highly expressed in apical membrane domains of particularly the late DCT2, with a gradual decrease along CNT and initial collecting duct (iCD) [13,14], suggesting that TRPV5-mediated Ca\(^{2+}\) uptake predominates in the DCT2 segment. Similarly, in rat, immunohistochemical data suggests that TRPV5 expression appears in the DCT2 and is further observed in the CNT [15]. The intrarenal distribution of TRPV5 in human is currently not known.

In comparison to rabbit, the boundaries defining the DCT/CNT/iCD regions in human, rat and mouse are morphologically less well defined, with cell types from different segments intermingled. In the kidney of rats, the DCT/CNT/iCD regions of rats and mice, and whether the CT-induced increase in cAMP could lead to a similar activation in the DCT2 segment as PTH.

In order to better understand the molecular actions of CT on overall renal Ca\(^{2+}\) balance and to delineate the potential effects of CT on TRPV5-dependent Ca\(^{2+}\) reabsorption, several experimental series were performed in wild-type and TRPV5-deficient mice. Due to the rapid action of CT, we investigated the short-term effects by of CT on the renal Ca\(^{2+}\) excretion.

Materials and methods

Experimental protocol 1

Male C57BL/6 mice (12 weeks of age) were housed in a light and temperature-controlled room with ad libitum access to deionized drinking water and standard chow (0.28% (wt/wt) NaCl, 1.00% (wt/wt) Ca, 0.22% (wt/wt) Mg; LabDiet, USA). Mice were injected subcutaneously with a single dose of salmon CT (20 U/100 g bodyweight, Novartis Pharmaceuticals, Taiwan, n = 6) or vehicle (n = 6) and placed in metabolic cages. After 16 hrs, overnight urine was collected, and the animals were sacrificed under halothane anesthesia. Blood was obtained via orbital puncture before cervical dislocation. The kidneys were dissected out and snap frozen for RNA extraction. The animal ethics boards of the National Defense Medical Center (Taipei, Taiwan) approved all animal experimental procedures.

Experimental protocol 2

TRPV5\(^{−/−}\) mice were generated by targeted ablation of the TRPV5 gene as described previously [20]. After acclimatization, TRPV5\(^{−/−}\) and TRPV5\(^{+/−}\) mice received salmon CT (20 U/100 g bodyweight, n = 6) or vehicle (n = 6) in three subcutaneous bolus injections spaced over 40 hrs. During this period, the mice were housed in metabolic cages. Overnight (16 hrs) urine was collected during the period between the first and second dose of CT. Subsequently, 24-h urine was collected during the remainder of the study, where the mice received the last two injections. At the end of the experiment, animals were killed under halothane anesthesia, blood was removed, and tissue samples were harvested for immunohistochemistry and western blotting. The animal ethics board of the Radboud University Nijmegen approved the animal experimental procedures.

Urine and serum analyses

Urine and serum concentrations of Ca\(^{2+}\) were analysed using a colorimetric assay kit (Roche, Mannheim, Germany). Urine and serum concentrations of Na\(^{+}\) and K\(^{+}\) were determined using an automated analyser (AU 5000 chemistry analyser, Olympus, Tokyo, Japan).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from kidney using Trizol Total RNA Isolation Reagent (Sigma, St Louis, MO, USA) as described previously [21]. The obtained total RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 1.5 µg of total RNA was reverse transcribed by Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). The cDNA was used to determine intrarenal mRNA expression levels of TRPV5, calbindin-D\(_{28K}\) (CaBP28K) and TRPV6 by quantitative real-time PCR, using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). The expression level of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as an internal control to normal-
Data is presented as mean ± SEM.

Immunohistochemistry
Kidney tissue was immersion fixed in 1% (wt/vol) periodate–lysine–paraformaldehyde for 2 hrs at room temperature, and subsequently incubated overnight at 4°C in phosphate-buffered saline (PBS) containing 15% (wt/vol) sucrose. The kidneys were snap frozen in liquid nitrogen and 7-μm sections cut on a cryostat microtome (Microm HM 550, MICROM International GmbH, Germany). For immunohistochemical detection of TRPV5, kidney sections were stained with a guinea pig anti-TRPV5 antibody (1:50) [8] or mouse anti-CaBP28K antibody (1:1000; Sigma). To visualize TRPV5 and CaBP28K, sections were stained with a guinea pig anti-TRPV5 antibody (1:50) [8] or mouse anti-CaBP28K antibody (1:1000; Sigma). To visualize TRPV5 and CaBP28K, sections were stained with goat anti-TRPV5 antibody (1:5000; Sigma) at 4°C. Subsequently, blots were incubated with a goat anti-IgG (1:300; Sigma), respectively. TRPV5 protein expression was semi-quantified by taking five digital images of each kidney section on with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY, USA) and calculating the integrated optical density using Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD).

Immunoblotting
Total mouse kidney lysates were prepared as described previously [23]. The protein concentration of the homogenates was determined with the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Samples were submitted to SDS–PAGE and blotted to polyvinylidifluoride-nitrocellulose membranes (Immobilon-P, Millipore Corp., Bedford, MA). Blots were incubated overnight with a rabbit anti-CaBP28K polyclonal antibody (1:5000; Sigma) at 4°C. Subsequently, blots were incubated with a goat anti-rabbit peroxidase-labelled secondary antibody (1 hr; 1:10000; Sigma, St. Louis, MO). Immunoreactive protein was detected by the chemiluminescence method (Pierce, Rockford, IL). Immunopositive bands were scanned using an imaging densitometer (Bio-Rad Gs-690) to determine pixel density (Molecular Analyst Software; BioRad Laboratories, Hercules, CA).

Table 1. Serum concentrations and urinary excretion of Na⁺ and K⁺ after a bolus injection CT

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<td>Na⁺ (mM)</td>
<td>K⁺ (mM)</td>
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<tr>
<td>Control</td>
<td>150 ± 0.4</td>
<td>5.34 ± 0.11</td>
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<tr>
<td>CT</td>
<td>152 ± 0.5</td>
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*P < 0.05 versus controls; CT, calcitonin.

Results

CT increases renal Ca²⁺ reabsorption in C57BL/6 mice
To assess the acute effects of CT, 16-hr urine from overnight collections was obtained following a bolus injection of the hormone (20 U/100 g body weight) or vehicle, respectively. The effect of overnight CT exposure on renal and systemic Ca²⁺ handling is depicted in Figure 1. Administration of CT resulted in a significant decrease in the urinary excretion of Ca²⁺ (P < 0.05). Serum Ca²⁺ concentrations were slightly reduced after overnight CT administration (P < 0.05). Urinary excretion of Na⁺ and K⁺ showed a significant decrease after CT injection (Table 1). In addition, the urinary Na⁺/K⁺ ratio remained unchanged excluding an effect on the collecting duct. These data are consistent with a primary effect of CT on electrolyte transport in the TAL and DCT [5–7,17].

CT does not change the renal mRNA expression of TRPV5, CaBP28K and TRPV6
To evaluate whether the increased Ca²⁺ reabsorption could be due to transcriptional changes in Ca²⁺ transport proteins located in the distal convolutions, quantitative real-time PCR was used to estimate TRPV5, CaBP28K and TRPV6 abundance. As can be seen in Figure 2, no changes were observed in the renal mRNA expression of these transcripts. Although CT did not modulate the changes of TRPV5, it does not exclude the potential involvement of TRPV5. Therefore, we investigated the effect of CT in TRPV5−/− and TRPV5−/+ mice after one and three injections of CT.

CT stimulates Ca²⁺ reabsorption in TRPV5−/− mice
Administration of CT to TRPV5−/− and TRPV5−/+ mice resulted in a decreased overnight urinary Ca²⁺ excretion in both groups. However, after repeated administration of CT, the urinary Ca²⁺ excretion returned to normal in both TRPV5−/− and TRPV5−/+ mice, as compared to their corresponding ve-
hicle-injected controls (Figure 3). Serum Ca\textsuperscript{2+} concentrations were measured after 40 hrs, but no difference was observed after CT administration in either strain (Figure 3). This is in line with the observed rapid effect of CT.

**Renal protein expression of Ca\textsuperscript{2+} transporters remains unaltered after three CT injections**

Kidneys retrieved from TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{−/−} mice by the end of the experiment were used to assess the effects of CT on renal Ca\textsuperscript{2+} transporter expression. TRPV5 and CaBP28K protein abundance was semi-quantified by immunohistochemistry (Figure 4) and immunoblotting (Figure 5), respectively. Computerized analysis of immunohistochemical images did not reveal any changes in TRPV5 protein expression after CT administration in TRPV5\textsuperscript{+/+} mice compared to their vehicle-injected control TRPV5\textsuperscript{+/+} mice (Figure 4). This is in line with the observation that CT affects renal Ca\textsuperscript{2+} transport independent of TRPV5. In addition, the renal CaBP28K protein expression remained unchanged after CT administration in TRPV5\textsuperscript{−/−} mice (Figure 4 and 5). The CaBP28K protein expression of TRPV5\textsuperscript{−/−} mice was significantly reduced compared to TRPV5\textsuperscript{+/+} mice [20] (Figure 4 and 5).
Discussion

A bolus injection of CT significantly reduced the urinary excretion of Ca\textsuperscript{2+} in wild-type mice. This decrease was associated with a reduction in the urinary Na\textsuperscript{+} and K\textsuperscript{+} excretion, leaving the urinary Na\textsuperscript{+}/K\textsuperscript{+} ratio unchanged. These data are consistent with the previously reported effects of CT on stimulating TAL and DCT transport of NaCl and K\textsuperscript{+} [6,7,17,24]. Earlier studies in the TAL of mice and rats have demonstrated that CT activates NaCl transport only in the cortical TAL [6,24]. This segment of the TAL is also thought to drive paracellular Ca\textsuperscript{2+} reabsorption in these species. Al-

![Fig. 4. Immunohistochemical staining of renal Ca\textsuperscript{2+} transporters after three bolus injections in TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{−/−} mice [representative images of immunohistochemical staining of TRPV5 (A) and CaBP28K (B) in kidney cortex; semi-quantification of renal TRPV5 (C) and CaBP28K (D) protein abundance was performed by computerized analysis of immunohistochemical images; data were calculated as integrated optical density (IOD; arbitrary units) and depicted as percentage of TRPV5\textsuperscript{+/+} controls; *P < 0.05, statistically significant; data is presented as mean ± SEM; n = 6 animals per group].
though never delineated in detail, CT is likely to activate a cAMP cascade leading to increased NKCC2 transport, perhaps via increased membrane trafficking and phosphorylation [5,6,25]. Currently, it remains unknown which transporter CT stimulates in the DCT [17]. One option is the thiazide-sensitive NaCl cotransporter that resides there.

A significant, albeit small, change in serum Ca²⁺ concentrations was observed after overnight CT administration. Earlier reports show that infusion of CT, depending on dose, acutely reduces serum Ca²⁺ concentrations that frequently revert to normal range within 1 day [26]. In line with this, no change was observed in systemic Ca²⁺ concentrations after three injections of CT spaced over 40 hrs. One may suggest that the reduced excretion of Ca²⁺ in the presence of CT occurs to compensate for the acute surge in systemic Ca²⁺ concentrations normally associated with CT administration. However, in thyroparathyroidectomized (TPTX) rats, Ca²⁺ infusion prior to CT treatment was given in order to avoid hypocalcaemia in the animals. In these TPTX rats, CT was still able to reduce the fractional excretion of Ca²⁺ despite normocalcaemia [27]. This is consistent with a direct effect of CT on the kidney. The effect of CT on urinary Ca²⁺ excretion is also absent after repeated administration of CT to either TRPV5+/+ or TRPV5⁻/⁻ mice. Reduced surface expression of CT receptors and decreased mRNA expression has been shown to explain such escape phenomena in other cell types [28]. This may also explain why patients with a CT-secreting tumour frequently have normal systemic concentrations of Ca²⁺ [29].

The present study was initiated to investigate the potential effect of CT on TRPV5-mediated Ca²⁺ reabsorption. Injections of CT for 16 or 40 hrs did not change the renal abundance of TRPV5. In addition, TRPV5⁻/⁻ and
TRPV5+/− mice responded in a similar manner to CT administration. Given the data obtained in these experimental models, it is likely that the effect of CT on renal Ca2+ transport occurs independently of TRPV5. Although CT does not affect TRPV5-mediated Ca2+ reabsorption, it is clear from this study that CT strongly stimulates renal Ca2+ reabsorption. The stimulatory effect of CT is likely to occur primarily through changes in Ca2+ transport, as previously described [6,7,24]. It should be stated that although the effects of CT on renal Ca2+ transport are present in TRPV5+/− as well as in TRPV5−/− mice, and that TRPV5 expression is unaltered after CT administration, this does not fully exclude an effect upon TRPV5. Although unlikely, a lack of stimulation of CT on TRPV5 in TRPV5−/− mice may be masked due to the pronounced effect of CT in the TAL. TRPV6 is a highly selective Ca2+ channel which has been localized to apical membrane domains in kidney [30]. As TRPV6 may play a role in apical Ca2+ entry of tubules in the distal part of the nephron, mRNA abundance of the channel was determined after an overnight bolus injection of CT. Administration of CT did not change the expression level of renal TRPV6. These data indicate that the effect of CT on renal Ca2+ transport occurs independently of TRPV6.

In the rat, CT has been shown to increase NaCl as well as Ca2+ transport in the DCT [17], thus vectorial transfer of Ca2+ does occur to a greater extent in the presence of CT. The present study suggests that these effects occur largely independent of TRPV5. In addition, microperfusion experiments in rabbits show that CT stimulates Ca2+ transport in the early DCT, where TRPV5 is not expressed [10]. This raises the question, how does Ca2+ transport occurs in the early DCT and does it contribute significantly to overall renal Ca2+ handling? Further studies are needed to determine the potential effect of CT on electrolyte transporters in these segments.

In conclusion, overnight CT administration increases renal Ca2+ reabsorption in mice. This effect occurs independently of TRPV5 as no change can be detected in TRPV5 and CaBP28K expression and similar responses to CT is observed in TRPV5+/− and TRPV5−/− mice.

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Conflict of interest statement. None declared.

References

**Introduction**

Rhabdomyolysis is a syndrome involving the breakdown of skeletal muscle, which causes myoglobin and other intracellular proteins and electrolytes to leak into the circulation [1]. It is often complicated by acute kidney injury (AKI), electrolyte imbalance and disseminated intravascular coagulation. About 10 to 50% of patients suffering from significant rhabdomyolysis develop some degree of AKI [2]. Although the treatment has been much improved, the mortality rate may still be as high as 8% [1,3,4]. The experimental model for rhabdomyolysis is easily acquired by injecting glycerol intramuscularly into rats or mice [5].

AKI by rhabdomyolysis has three pathogenic mechanisms: tubular obstruction, renal vasoconstriction and oxidative stress. oxidative stress has been an important target in the prevention of myoglobin-induced renal injury [6]. The administration of antioxidants has been shown to provide partial protection against myoglobinuric-induced AKI [7–11]. N-acetylcysteine (NAC), one of these antioxidants, is a source of sulfhydryl and glutathione (GSH) groups in cells and, due to its interaction with reactive oxygen species, is a scavenger of free radicals [12]. The protective effect of NAC with respect to renal injury has been proven in various models, such as cisplatin [13], ischaemia–reperfusion injury [14,15] and chronic kidney disease [16]. However, there is little data for administering NAC in the rhabdomyolysis model, and the results are controversial [17,18].