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Impaired renal calcium absorption in mice lacking calcium channel ß$_3$ subunits

José F. Bernardo[*], **Clara E. Magyar**[†], **W. Bruce Sneddon**[†], and **Peter A. Friedman**[*,†]

[*]Department of Medicine, Renal Electrolyte Division, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

[†]Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Abstract

Transgenic mice lacking calcium channel ß$_3$ subunits (Ca_Vß$_3$) were used to determine the involvement of a multimeric calcium channel in mediating stimulated renal calcium absorption. We measured the ability of calcium channel ß$_3$ subunit-null (Ca_Vß$_3^{-/-}$) and wild-type (Ca_Vß$_3^{+/+}$) mice to increase renal calcium absorption in response to the calcium-sparing diuretic chlorothiazide (CTZ). Control rates of fractional sodium excretion were comparable in Ca_Vß$_3^{-/-}$ and Ca_Vß$_3^{+/+}$ mice and CTZ increased sodium excretion similarly in both groups. CTZ enhanced calcium absorption only in wild-type Ca_Vß$_3^{+/+}$ mice. This effect was specific for diuretics acting on distal tubules because both Ca_Vß$_3^{-/-}$ and Ca_Vß$_3^{+/+}$ mice responded comparably to furosemide. The absence of ß$_3$ subunits resulted in compensatory increases of TrpV5 calcium channels, the plasma membrane Ca-ATPase, NCX1 Na/Ca exchanger protein, and calbindin-D_{9k} but not calbindin-D_{28k}. We conclude that TrpV5 mediates basal renal calcium absorption and that a multimeric calcium channel that includes Ca_Vß$_3$ mediates stimulated calcium transport.

Keywords

ion channels; calcium homeostasis; calcium transport; transgenic mice; diuretics

Introduction

Extracellular calcium homeostasis is maintained by integrated intestinal calcium absorption and renal calcium excretion. In normal human adults, most calcium filtered by the kidneys is reabsorbed, and only a small fraction, which is equal to the amount absorbed by the intestines, is excreted into the urine to maintain calcium balance. The majority of the filtered calcium is recovered by proximal tubules. However, it is in the more distal nephron segments, including cortical thick ascending limbs and distal convoluted tubules, where the fine control of calcium recovery occurs. Parathyroid hormone (PTH) enhances calcium absorption by both cortical ascending limbs and distal convoluted tubules, while 1,25$(OH)_2$ vitamin D_3 facilitates PTH-dependent calcium transport in distal convoluted tubules (Friedman & Gesek, 1993; Gesek & Friedman, 1992b). Pharmacologic agents such as chlorothiazide (CTZ) also stimulate calcium

correspondence: Peter A. Friedman, Ph.D., Univ. of Pittsburgh School of Medicine, Dept. of Pharmacology & Chemical Biology, W1340 Biomedical Science Tower, Pittsburgh, PA 15261 USA, phone: 412-383-7783, FAX: 412-648-1945, paf10@pitt.edu.

Dr. Magyar's present address is: Department of Pathology and Laboratory Medicine, The David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.

Dr. Sneddon's current address is: Department of Biological Sciences, Duquesne University, Pittsburgh, PA 15282.

absorption by distal convoluted tubules (Costanzo, 1985; Edwards, Baer, Sutton, & Dirks, 1973; Ellison, Velázquez, & Wright, 1987) and distal convoluted tubule cells (Friedman & Gesek, 1993). The net result of this reabsorptive process is that less than one percent of the filtered calcium is excreted in the voided urine. Under normal conditions, renal calcium handling is the key physiological response that maintains calcium balance (Stewart, 1992).

In distal convoluted tubules, PTH and CTZ stimulate active transcellular calcium absorption (Gesek & Friedman, 1992a, 1992b). Transcellular calcium movement is a three-step process. Calcium enters the cell across apical plasma membranes, diffuses through the cytoplasm, and is extruded by energy-dependent process across the basolateral cell membranes. The mechanism of apical membrane calcium entry in distal convoluted tubules is uncertain. Calcium influx in immortalized mouse distal convoluted tubule cells proceeds through a dihydropyridine-sensitive mechanism (Barry, Gesek, Yu, Lytton, & Friedman, 1998; Friedman & Gesek, 1993) that is mediated by a 2-pS calcium channel (Matsunaga, Stanton, Gesek, & Friedman, 1994). Calcium transport is stimulated by PTH and calcitonin, and by CTZ and amiloride (Friedman & Gesek, 1993; Gesek & Friedman, 1992a, 1992b), and blocked by nimodipine. In the absence of a stimulus, calcium entry and current are negligible (Bacskai & Friedman, 1990; Friedman & Gesek, 1993; Matsunaga et al., 1994).

Voltage-dependent Ca^{2+} channels (Ca_V) consist of a pore-forming α1subunit that associates with ß, γ, and δ subunits (Ca_Vß, Ca_Vγ, Ca_Vδ). β subunits are cytosolic and exert marked effects on $Ca_V\alpha_1$ function, including trafficking of Ca^{2+} channel complexes to the plasma membrane, voltage-dependence and activation/inactivation kinetics of Ca^{2+} currents (Berrow, Campbell, Fitzgerald, Brickley, & Dolphin, 1995; Dolphin, 2003). Four β subunits have been cloned. Although the molecular identification of the oligomeric kidney calcium channel is uncertain it appears to be a multimeric protein that includes a Ca_Vß subunit (Yu et al., 1995). In previous work we localized $Ca_V ß_3$ and $Ca_V\alpha_{1c}$ to mouse distal tubule cells. A key role for $Ca_V ß_3$ in cellular calcium entry was determined because antisense deoxyoligonucleotides targeted to the $Ca_V ß_3$ sequence selectively blocked calcium transport induced by PTH or by CTZ (Barry et al., 1998). The calcium channel blocker nifedipine also inhibited calcium absorption by distal tubules of the rat (Kauker, Zawada, Kauker, Roman, & Rosivall, 1997). In contrast to these findings, a calcium-selective epithelial calcium channel, TrpV5, has been cloned and localized to the apical membrane of late distal tubules (Vennekens et al., 2000). TrpV5 is a homotetramer that is constitutively active, has a 77 pS single channel conductance (Nilius et al., 2000), is insensitive to dihydropyridine or to the phenylalkylamine, verapamil, calcium channel blockers (J. G. J. Hoenderop et al., 1999; Muller, Hoenderop, van Os, & J. M. Bindels, 2001; Vennekens et al., 2000). No stimulatory effect of PTH or of CTZ on calcium transport mediated by TrpV5 has been reported.

In the present study we conducted experiments to determine the role $Ca_V ß_3$ on renal calcium conservation *in vivo*. This was accomplished by measuring renal calcium reabsorption under resting conditions and after a calcium-sparing challenge in mice lacking $Ca_V ß_3$ (Namkung et al., 1998). The results show that $Ca_V ß_3$-null mice have a blunted calcium-sparing response to CTZ but normal resting calcium metabolism. The findings implicate a role for a multimeric calcium entry channel in mediating calcium absorption by distal convoluted tubules.

Materials and Methods

Animals and genotyping

Experimental protocols used in this study were carried out in accord with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the IACUC of the University of Pittsburgh School of Medicine. Original heterozygous $Ca_V ß_3^{+/-}$ breeding pairs were provided by Professor Hee-Sup Shin, Pohang University of Science and Technology, Republic

of Korea. Male wild-type ($Ca_V ß_3^{+/+}$), and β_3-null ($Ca_V ß_3^{-/-}$) mice weighing 20–30 g with free access to standard rodent diet (Prolab RMH 3000, Richmond, IN) and tap water, were used for the experiments. PCR of genomic DNA used: S primer (5′-TGGACCGGATCTTCACAGCG-3′), A2 primer (5′-GGATGCAGAACACGGCTAGT-3′), and PGK primers (5′-CTGACTAGGGGAGGAGTAGAAG-3′). The wild-type locus yields a 452-bp fragment amplified by S and A2 primers, whereas the mutant locus yields a 290-bp fragment amplified by the A2 and PGK primers (Namkung et al., 1998).

Electrolyte measurements in conscious mice

Mice were individually housed in metabolic cages (Nalgene, Rochester, NY) with free access to food and water. After an acclimation period of seven days, spontaneously voided urine was collected daily under mineral oil for seven additional days for determination of electrolyte excretion. Urinary sodium, calcium, and pH were measured using a Medica EasyLyte Ca/Na/K/pH Analyzer (Bedford, MA).

Clearance protocol

The animals were prepared for clearance experiments according to conventional techniques as modified for use in the mouse (J. N. Lorenz & Gruenstein, 1999; J.N. Lorenz, Schultheis, Traynor, Shull, & Schnermann, 1999). Mice were anesthetized with separate intraperitoneal injections of ketamine (50 μg/g body wt) and thiobutabarbital (Inactin™, 100 μg/g body wt, Sigma) and placed on a thermally controlled surgical table. Following tracheostomy, the right femoral artery and jugular vein were cannulated with polyethylene tubing hand-drawn over a flame to a fine tip (0.3–0.5 mm OD). The arterial catheter was connected to a fixed-dome pressure transducer (DIGI-MED, Louisville, KY) for measurement of arterial blood pressure. This catheter was also used for blood sampling. Blood pressure and heart rate were monitored continuously every 4 min using a data acquisition system. The venous catheter was connected to a syringe pump for infusion. After the venous catheter had been placed, a maintenance infusion of isotonic saline containing 2.25 g bovine serum albumin, 1 g glucose, and 0.75 g FITC-inulin/100 ml was administered at 0.25μl/(min × g body weight) throughout the experiment. Previous studies established that FITC-inulin yields measurements of GFR indistinguishable from radioactive inulin (J. N. Lorenz & Gruenstein, 1999; J.N. Lorenz et al., 1999). The bladder was cannulated with flared PE-10 polyethylene tubing for the collection of urine. Body temperature was maintained at 37.5°C, and animals were suffused with a steady stream of 100% O_2. Following surgery, a period of 45–60 min was allowed for stabilization. The experiment consisted of 4 periods of 30-min duration each. During the first two periods (C1 and C2), baseline parameters were obtained. Following this, CTZ was administered as a 2 mg/kg bolus followed by a continuous intravenous infusion of 0.25 mg/(kg × min). In separate experiments, furosemide (FUR) was administered as a 10 mg/kg bolus followed by a continuous intravenous infusion of 1 mg/(kg × min). After a 10-min equilibration following the diuretic infusion, two additional periods of 30 min each were collected. Urine was collected during each of the 4 periods, and a midpoint blood sample (30 μl) was taken. Two additional 40-μl blood samples were obtained at a midpoint in the first and fourth period for determination of pH, sodium, potassium, calcium, and hematocrit (Rapidlab 348, Bayer Diagnostics, Tarrytown, NY). Urine output was determined gravimetrically. To avoid changes in blood pressure and shock, the animals received an equivalent volume of whole blood from mice donors. Blood samples were centrifuged, and plasma aliquots were used for duplicate inulin determinations. Inulin was also measured in diluted (1:100) urine samples for the determination of glomerular filtration rate (GFR). Briefly, 3-μl samples of plasma or diluted urine were dispensed into 197 μl of 10 mM HEPES buffer in 96-well microplates. The fluorescence was read using a microplate fluorometer with an excitation wavelength of 485 nm and an excitation wavelength of 538 nm.

GFR, sodium, potassium, and calcium clearance were calculated by standard formulas. Fractional solute excretion (FE_x) was calculated as $Cl_x/Cl_{In}x$ 100, where Cl_x is the clearance of the x^{th} solute and Cl_{In} is the inulin clearance. Calcium reabsorption (T_{Ca}, nmol/min) was calculated as the difference between the amount of calcium filtered ($GFR \times P_{Ca}$) and the amount excreted (UCa). The rate of urinary sodium, and potassium excretion were expressed as μmol/min, and calcium reabsorption as nmol/min. The two control periods and the two experimental periods were averaged and comparisons were made between the two conditions.

Immunoblot analysis

Whole kidney lysates were used to analyze cytoplasmic proteins calbindin-D_{9k} and calbindin-D_{28k}. Kidneys were minced and homogenized in 2 ml of ice-cold isolation buffer (5% sorbitol, 0.5 mM Na_2EDTA, 5 mM histidine-imidazole buffer with 0.2 mM phenylmethylsulfonylfluoride (PMSF), 1.4 μM aprotinin, pH 7.5) with a Teflon dounce homogenizer. Kidney membranes were used to analyze membrane-delimited proteins (TrpV5, NCX1, PMCA). Membranes were isolated by centrifuging 1 ml of the whole kidney homogenate at 2,000 × g for 10 min. The supernatant, containing total membranes and soluble proteins, was pelleted by centrifugation (100,000 × g for 1.5 h) and resuspended in 1ml of isolation buffer. Total protein concentrations were measured (Bio-Rad Dc Protein Assay). Constant amounts of protein from either homogenate or membrane samples (solubilized in Laemmli sample buffer) were resolved on 5% or 7.5% polyacrylamide gels by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore) according to standard methods. Membranes were blocked overnight at 4°C with 5% nonfat dried milk in Tris-buffered saline + Tween® 20 (TBST) (Sigma), incubated with respective primary antibodies for 4 h at room temperature, washed, and incubated with appropriate secondary antibodies for 1 h at room temperature. Protein bands were visualized with a luminol-based enhanced chemiluminescence substrate (Santa Cruz Biotechnology, Inc.) and band densities were analyzed with NIH Image 1.61 (http://rsb.info.nih.gov/nih-image). Parallel gels were run and stained with Coomassie Blue to verify protein loading. Polyclonal primary antibodies against the rabbit $Ca_V ß_3$ and the epithelia Na channel (ENaC) alpha subunit were obtained from Chemicon International (Temecula, CA). Rat TrpV5 antiserum was from Alpha Diagnostic International (San Antonio, TX). Its specificity was validated using the peptide determinant of TrpV5. Canine Na+/Ca2+ exchanger-1 (NCX1) antiserum was from Swant (Bellinzona, Switzerland). A monoclonal antibody against the plasma membrane Ca^{2+}-ATPase (PMCA) was purchased from Affinity Bioreagents [ABR] (Golden, CO) was used at 1:1000 dilution. Anti-calbindin-D_{28k} polyclonal antibody (1:10,000 was from (Sigma, St. Louis, MO, and anti–calbindin-D_{9K} polyclonal antibody (1:5000) was from Swan. Secondary antibodies, goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce Biotechnology, Rockford, IL) were used at 1:5000.

Statistical analysis

The data are presented as means ± SE, where n indicates the number of independent experiments. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as the mean ± SE of n independent experiments. Paired results were by Student t-test (Prism; GraphPad, San Diego, CA). Comparisons of slopes were analyzed by ANOV. Differences lower than $P \leq 0.05$ were assumed to be significant.

Results

We first confirmed that $Ca_V ß_3$ was expressed by kidneys of wild-type $Ca_V β_3^{+/+}$ but not $Ca_V β_3^{-/-}$ mice (Fig. 1). A synthetic peptide consisting of residues 463–477 of the rabbit $Ca_V ß_3$ subunit (GenBank Accession number P54286) virtually abolished staining (not shown).

Notably, the epithelial Na channel, ENaC, which is expressed at apical cell membranes of distal nephrons, was equivalently expressed in $Ca_V\beta_3^{+/+}$ and $Ca_V\beta_3^{-/-}$ mice (Fig. 1). This finding substantiates the localization of Ca_Vß$_3$, the specificity and completeness of $Ca_V\beta_3$ knockout, and that the membrane preparation is free from detectable contamination.

Body weight, MAP and GFR were comparable for the two groups of mice. Baseline serum parameters for $Ca_V\beta_3^{+/+}$ and $Ca_V\beta_3^{-/-}$ mice are given in Table 1. Serum Na^+, Ca^{2+}, and K^+ were indistinguishable in $Ca_V\beta_3^{+/+}$ and $Ca_V\beta_3^{-/-}$ mice. Control rates of urine flow, absolute and fractional urinary sodium excretion, and calcium reabsorption were also similar between the two groups. These findings suggest the absence of conspicuous differences in calcium homeostasis under static conditions. Examination of the dynamic relations between sodium and calcium excretion is shown in Fig. 2. Here, basal rates of calcium excretion were comparable in both mouse strains. However, at elevated rates of Na^+ excretion, Ca^{2+} elimination was greater in $Ca_V\beta_3^{-/-}$ mice than in wild-type $Ca_V\beta_3^{+/+}$ animals ($P < 0.01$), indicating an underlying deficiency of renal calcium absorption in the absence of Ca_Vß$_3$.

To uncover such a deficiency, we determined if mice lacking $Ca_V\beta_3^{-/-}$ could mount a calcium-sparing response to challenge by CTZ, which exerts its diuretic and calcium-sparing actions uniquely on distal convoluted tubules. We compared the effects of CTZ on fractional Na excretion (FE_{Na}) and on calcium reabsorption (T_{Ca}) in $Ca_V\beta_3^{+/+}$ and $Ca_V\beta_3^{-/-}$ null mice. Control rates of FE_{Na} were comparable in the two groups and CTZ increased FE_{Na} similarly in both groups of mice (Fig. 3). Strikingly however, CTZ augmented T_{Ca} only in wild-type $Ca_V\beta_3^{+/+}$ mice. These results establish that renal distal tubules were fully responsive to the natriuretic action of CTZ and that the failure to increase calcium reabsorption cannot be attributed to a structural or general loss of normal function of the cells forming the distal convoluted tubule (Loffing et al., 1996).

Because renal calcium absorption and sodium excretion change dynamically, we further analyzed the relations between these two parameters. Fig. 4 displays the relationship between paired measurements of FE_{Na} and FE_{Ca} under resting conditions and following CTZ infusion in wild-type $Ca_V\beta_3^{+/+}$ mice (top) and $Ca_V\beta_3^{-/-}$ null mice (bottom). Under resting conditions there was a significant linear relationship between FE_{Ca} and FE_{Na}, that was displaced to the right after CTZ in $Ca_V\beta_3^{+/+}$ mice but not in $Ca_V\beta_3^{-/-}$ mice. CTZ lowered calcium excretion at any given level of sodium excretion, changing the slopes significantly ($P<0.001$) uniquely in $Ca_V\beta_3^{+/+}$. These findings point to a requirement for calcium channel Ca_Vß$_3$ in mediating the calcium-sparing action of CTZ.

To determine if the diuretic and calcium-sparing actions were specific to CTZ, similar experiments were performed with furosemide (FUR), a drug that blocks Na-K-2Cl cotransport in thick ascending limbs and causes parallel increases of Na^+ and Ca^{2+} excretion. FUR significantly increased V, $U_{Na}V$, and FE_{Na}. The magnitude of these increases was similar in $Ca_V\beta_3^{+/+}$ and in $Ca_V\beta_3^{-/-}$ mice (Fig. 5). We next compared the dynamic actions of FUR on the relationship between urinary calcium and sodium excretion. The top panel in Fig. 6 shows the relationship between fractional calcium excretion as a function of fractional sodium excretion before and after FUR in $Ca_V\beta_3^{+/+}$ mice. The highly linear relationships between FE_{Ca} and FE_{Na} were not significantly altered by FUR administration in $Ca_V\beta_3^{+/+}$ or in $Ca_V\beta_3^{-/-}$ animals. Thus, the deficiency in calcium excretion in $Ca_V\beta_3^{-/-}$ mice is not attributable to a general change of renal calcium conservation.

Compensatory changes of calcium channel abundance in Ca_Vß$_3^{-/-}$ mice

Although distal tubule calcium absorption was compromised in Ca_Vß$_3^{-/-}$ mice, it was not abolished. This suggested the possibility of compensatory adaptation of alternate calcium transport mechanisms. Therefore, we inquired if ablation of Ca_Vß$_3$ leads to compensatory

changes in the abundance of TrvpV5, which mediates basal calcium transport. Fig. 7a shows a representative immunoblot from $Ca_V\beta_3^{-/-}$ and $Ca_V ß_3^{+/+}$ mice and Fig. 7b summarizes the relative protein abundance for 3 independent determinations. TrpV5 abundance nearly doubled in $Ca_V\beta_3^{-/-}$ compared to $Ca_V ß_3^{+/+}$ control mice ($P < 0.01$). Antibody specificity was validated by using a peptide determinant rat TrpV5, which abolished detection. We reasoned that if calcium entry mediated by TrpV5 increased in $Ca_V ß_3^{-/-}$ mice, then expression of proteins mediating calcium efflux might also increase to permit restoration of calcium absorption. The results shown in Fig. 7a and summarized in Fig 7b bear out this idea because expression of plasma membrane Ca^{2+}-ATPase, PMCA, and the Na^+/Ca^{2+} exchanger, NCX1, were greater in kidneys from $Ca_V ß_3^{-/-}$ animals than in wild-type controls. Calbindin-D_{9k} \expression increased substantially, while calbindin-D_{28k} expression was equivalent in $Ca_V ß_3^{-/-}$ and $Ca_V ß_3^{+/+}$ mice.

Discussion

The results described here define a role for $Ca_V\beta_3$ in mediating renal calcium conservation. Mice lacking $Ca_V\beta_3$ subunits express fewer N-type Ca^{2+} channels, impaired synaptic transmission (Namkung et al., 1998), and enhanced NMDA receptor-dependent long-term potentiation (Jeon et al., 2008). As shown here, serum calcium and baseline urinary calcium excretion were unaffected in these animals. There is no *a priori* reason to expect that basal serum calcium or urinary calcium excretion would be adversely affected in the absence of one of the distal nephron calcium entry proteins, especially under resting conditions. We hypothesized that if voltage activated-calcium channels (Ca_V) participate in mediating calcium entry in the distal nephron, this capacity should be impaired when the animals were challenged with a thiazide diuretic. Consistent with this view, $Ca_V ß_3^{-/-}$ mice were significantly impaired in their ability to mount a calcium-sparing response to pharmacological intervention that specifically stimulates calcium absorption by distal renal tubules (Costanzo & Weiner, 1976; Costanzo & Windhager, 1978). Thus, it is reasonable to conclude that resting serum calcium levels are defended at the expense of other regulatory parameters. Clearly, the absence of $Ca_V ß_3$ exerts a significant effect when animals were challenged. The present observations extend *in vitro* studies regarding the importance of $Ca_V ß_3$ in mediating PTH and CTZ-stimulated calcium transport. Those experiments delineated the involvement of $Ca_V\alpha 1c$ and $Ca_V ß_3$-containing calcium channels in mediating CTZ-stimulated calcium transport in distal tubule cells (Bacskai & Friedman, 1990; Barry et al., 1998; Matsunaga et al., 1994). PTH-stimulated calcium transport is mediated by calcium channels using the same $ß_3$ subunit but a different, and as yet undefined, $Ca_V\alpha$ subunit.

Renal handling of calcium and sodium are interdependent (Friedman, 1998). Because of this relationship between renal calcium and sodium handling, the assessment of the influence of any factor on urinary calcium excretion must take account of simultaneous changes in urinary sodium excretion (Sutton & Dirks, 1978). Normally, there is a linear relationship between the fractional excretion of calcium and sodium in humans and experimental animals (Costanzo, 1985; Costanzo, Moses, Rao, & Weiner, 1975; Edwards et al., 1973; Walser, 1971). Thiazide diuretics disrupt this relationship by increasing distal renal tubule calcium absorption and thereby reducing the clearance ratio of calcium/sodium (C_{Ca}/C_{Na}). This action is seen upon acute administration (Costanzo & Weiner, 1974) and during chronic drug treatment (Brickman, Massry, & Coburn, 1972; Parfitt, 1972). The acute administration of thiazide diuretics results in a marked natriuresis, but only a small increase, or even a decrease, in calcium excretion. This effect is due to dissociation of Na^+ and Ca^{2+} transport in distal tubules. This finding was borne out here in wild-type $Ca_V ß_3^{+/+}$ mice. However, $Ca_V ß_3^{-/-}$-null mice failed to respond in a comparable manner. $Ca_V ß_3^{-/-}$ mice animals, it should be noted, appear to exhibit lower FE_{Na} (less than 5% compared to many data points beyond 5% in $Ca_V ß_3^{+/+}$ mice). We attempted to compare FE_{Ca} using $Ca_V ß_3^{+/+}$ and $Ca_V ß_3^{-/-}$ mice over an expanded and similar range of

FE_{Na} by inducing a mannitol diuresis to extend the range of observed Na excretion. However, we were unable to match fluid repletion with the magnitude of diuresis and animals became dehydrated or fluid loaded. This disparity might contribute to the apparent reduced T_{Ca} response to CTZ.

Previous results from our laboratory support the view that apical membrane calcium entry in distal tubule cell culture models involves a multimeric, dihydropyridine-sensitive calcium channel (Barry et al., 1998). This channel is stimulated by PTH and by CTZ (Bacskai & Friedman, 1990; Matsunaga et al., 1994); is inactive at baseline; has a small, 2 pS, conductance, is activated at hyperpolarizing voltages; is blocked by dihydropyridine-type calcium channel blockers, and consists of $Ca_V\alpha$ and Ca_Vß subunits. These biophysical and physiological properties differ substantially from those of TrpV5 (Mensenkamp, Hoenderop, & Bindels, 2007). TrpV5, which is localized to late distal tubules and connecting tubules (J. G. J. Hoenderop et al., 1999), for instance, exhibits a single channel conductance of 77 pS (Nilius et al., 2000); is constitutively open, is apparently insensitive to CTZ or PTH, and is refractory to dihydropyridine or phenylalkylamine type calcium channel blockers (J. G. J. Hoenderop et al., 1999). It is formed as a homotetramer and lacks accessory subunits (J. G. Hoenderop et al., 2003). Ablation of TrpV5 channel causes renal calcium wasting (J. G. J. Hoenderop et al., 2003). The available evidence suggests that it is likely that several distinct Ca^{2+} channels are present in apical membranes of the distal nephron (J. G. Hoenderop, Willems, & Bindels, 2000). Based on the functional complementarity of these two calcium channels, and the upregulation of TrpV5 in animals lacking calcium channel ß$_3$ subunits, we propose that TrpV5 mediates basal calcium entry in distal tubule cells and that the multimeric calcium channel consisting of $Ca_V\alpha_1$ß$_3$ mediates CTZ-stimulated Ca^{2+} entry. Redundant Ca_Vß subunits are not expressed in distal tubules or cannot rescue the absence of Ca_Vß$_3$. It is likely that a different $Ca_V\alpha$ subunit, with as yet unknown identity, complexed with a common Ca_Vß$_3$ subunit is responsible for PTH-stimulated Ca^{2+} transport (Barry et al., 1998). Alternatively, we cannot rule out the possibility that multimeric Ca_V mediates calcium transport by early distal tubules, the so-called DCT1 segment, and that TrpV5 mediates this action in late distal tubule DCT2 segments.

Calcium absorption in thick ascending limbs is largely passive and is driven by the ambient electrochemical gradient, which is established primarily as a consequence of sodium absorption. Thus, increases of Na^+ absorption augment the transepithelial voltage and, in turn, stimulate calcium absorption. Conversely, decreases of Na^+ absorption are attended by decreases of Ca^{2+} absorption. The magnitude of natriuresis is generally paralleled by the calciuresis. Furosemide, a so-called loop diuretic that is a specific inhibitor of the Na^+/K^+2Cl^- cotransporter increased absolute and fractional sodium excretion equivalently in Ca_Vß$_3^{+/+}$ and Ca_Vß$_3^{-/-}$ mice. Absolute and fractional calcium excretion increased such that the FE_{Ca}/FE_{Na} ratio returned to, and slightly surpassed the initial value. In the present study, Ca_Vß$_3$ wild-type and null-mutant mice exhibited similar increases of urinary flow rate, urinary sodium excretion and FE_{Na} after furosemide. These findings suggest, therefore, that the Ca_Vß$_3$ does not influence the pharmacological response to blockade of the electroneutral $Na^+/K^+/2Cl^-$ cotransporter, and conversely that the effect of furosemide on calcium transport does not involve Ca_Vß$_3$. Such a conclusion is entirely consistent with the view that calcium absorption in thick ascending limbs proceeds predominantly through the lateral intercellular space and is driven by, and changes in parallel with, the magnitude of sodium absorption. More importantly, the baseline linear relationship between calcium excretion and sodium excretion was not modified by furosemide administration in either wild-type or Ca_Vß$_3$-null mice. Thus, the pharmacological response to CTZ and of distal convoluted tubules is specific and different in mice lacking Ca_Vß$_3$.

It is unlikely that $Ca_V ß_3$ subunits influence membrane potential. Indeed, partial Ca conductance (G_{Ca}) is too small to cause a meaningful effect on membrane voltage (Matsunaga et al., 1994). By extension, any voltage change would insignificantly alter the rate of Na/Ca exchange mediated by NCX1 (Friedman, 1998). However, administration of thiazide diuretics significantly hyperpolarizes distal tubule cells, thereby increasing Ca^{2+} entry (Gesek & Friedman, 1992a) and efflux, with an attendant decrease of Na^+ absorption (Friedman & Bushinsky, 1999). In the absence of $Ca_V ß_3$ this calcium-sparing action of thiazide diuretics is lost. These data are consistent with the view that $Ca_V ß_3$ absence in distal nephrons causes diminished apical uptake of Ca^{2+} that is accompanied by an increase of calcium excretion.

$Ca_V ß_3$ was uniformly knocked out in the animals studied here. Notably, mean arterial blood pressure and GFR were comparable in $Ca_V β_3^{+/+}$ and $Ca_V β_3^{-/-}$ mice. Hence, even if $Ca_V ß_3$ shown in Fig. 1 were derived from renal blood vessels its absence has no physiological impact on kidney function. In contrast, the loss of $Ca_V ß_3$ from tubular epithelial cells was specifically associated with impaired calcium conservation induced by CTZ. Furosemide action was entirely normal. These findings provide the first evidence for a physiological role of $Ca_V ß_3$ in renal calcium homeostasis.

Depletion of $Ca_V β_3$ leads to compensatory changes in the abundance of TrvpV5, which mediates basal calcium transport. Augmented apical calcium entry is tightly linked to the rate of basolateral membrane calcium efflux and, indeed, expression of proteins mediating cellular calcium efflux also increased. These latter processes are mediated by PMCA and NCX1. Calbindin-D_{9k} is linked to the regulation of PMCA by vitamin D (Christakos et al., 2007).

In summary, the present *in vivo* studies identify a specific role for multimeric calcium channels in mediating regulated calcium absorption by renal distal tubule cells. In this regard, the findings extend and substantiate the predictions based on *in vitro* cell culture models, where calcium transport was negligible under resting conditions and required the presence of functional calcium channel $ß_3$ subunits to respond to stimulation by PTH or CTZ (Barry et al., 1998; Matsunaga et al., 1994). Based on these findings we conclude that TrpV5 mediates basal renal calcium absorption and that a multimeric calcium channel that includes $Ca_V ß_3$ is necessary for stimulated renal calcium absorption.

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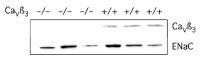

Figure 1.

$Ca_V ß_3$ and ENaC expression in kidney membranes of $Ca_V ß_3^{-/-}$ mice (lanes 1–3) and wild-type $Ca_V ß_3^{+/+}$ mice (lanes 4–6). Protein in each lane was isolated from a separate mouse. Immunoblotting details are described in Methods.

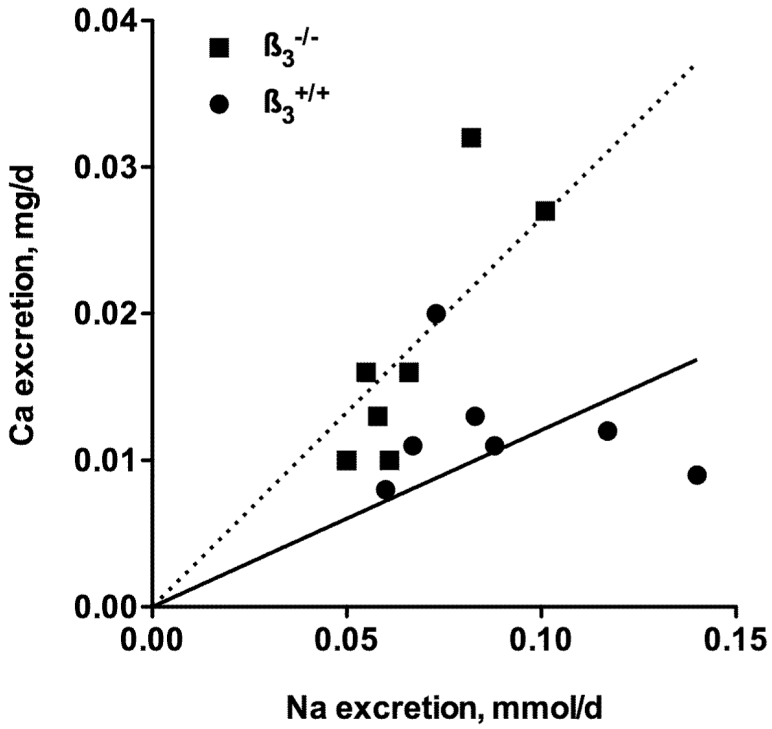

Figure 2.
Spontaneous calcium and sodium excretion in $Ca_V\beta_3$ wild-type (Ca $\beta_3^{+/+}$) and null-mutant ($Ca_V\beta_3^{-/-}$) mice. Electrolyte excretion was monitored in mice housed in metabolic cages.

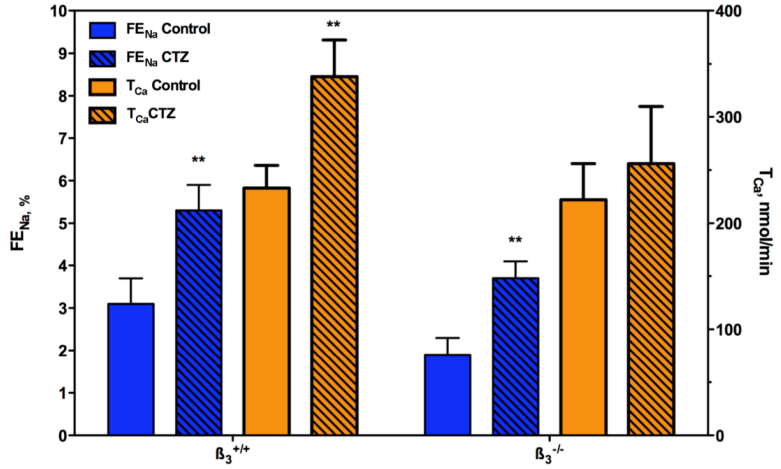

Figure 3.

Fractional sodium excretion (FE_{Na}, %) and calcium absorption (T_{Ca}) in β_3 wild-type ($Ca_V\beta_3^{+/+}$) and null-mutant mice ($Ca_V\beta_3^{-/-}$) before (control) and after chlorothiazide (CTZ) infusion (2 mg/kg bolus followed by a continuous intravenous infusion of 0.25 mg/[kg × min]). Baseline control values were not significantly different between groups. *P<0.01 in comparison to control.

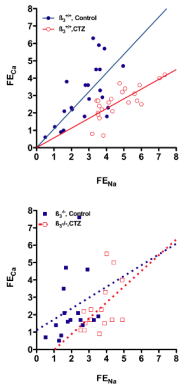

Figure 4.

Fractional calcium excretion (FE_{Ca}, %) as a function of fractional sodium excretion (FE_{Na}, %) in wild-type $Ca_V ß_3^{+/+}$ (upper panel) and null-mutant $Ca_V ß_3^{-/-}$ (lower panel) mice before (control) and after intravenous chlorothiazide (CTZ) infusion (2 mg/kg bolus followed by 0.25 mg/kg/min).

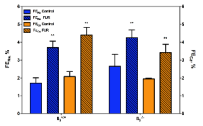

Figure 5.
Fractional sodium (FE_{Na}, %) and calcium (FE_{Ca}) excretion in β_3 wild-type ($Ca_V\beta_3^{+/+}$) and null-mutant mice ($Ca_V\beta_3^{-/-}$) before (control) and after furosemide (FUR) (10 mg/kg bolus followed by a continuous intravenous infusion at 1 mg/[kg × min].

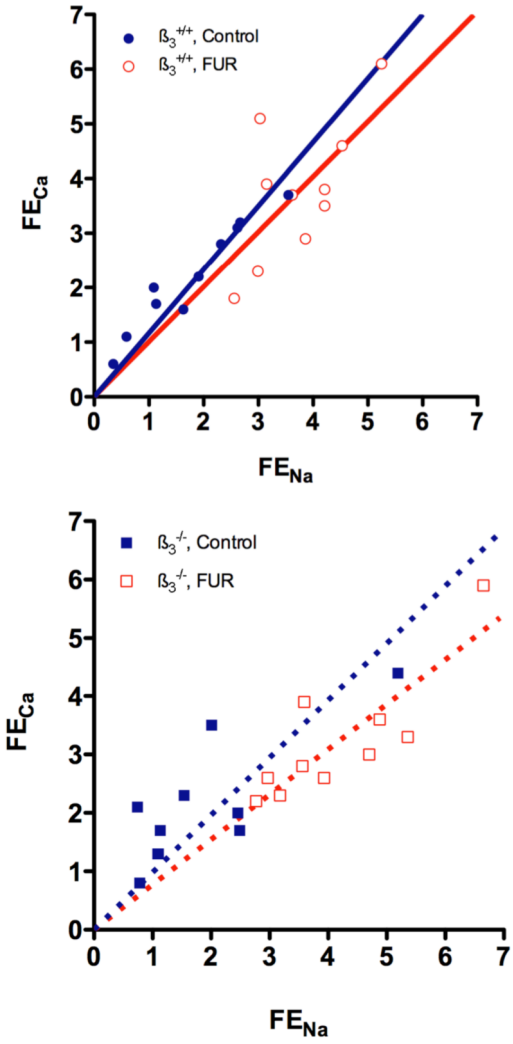

Figure 6.
Fractional calcium excretion (FE_{Ca}, %) as a function of fractional sodium excretion (FE_{Na}, %) in wild-type $Ca_V ß_3^{+/+}$ (upper panel) and null-mutant $Ca_V ß^{-/-}$ (lower panel) mice before (control) and after intravenous furosemide (FUR) infusion (10 mg/kg bolus followed by 1 mg/kg/min).

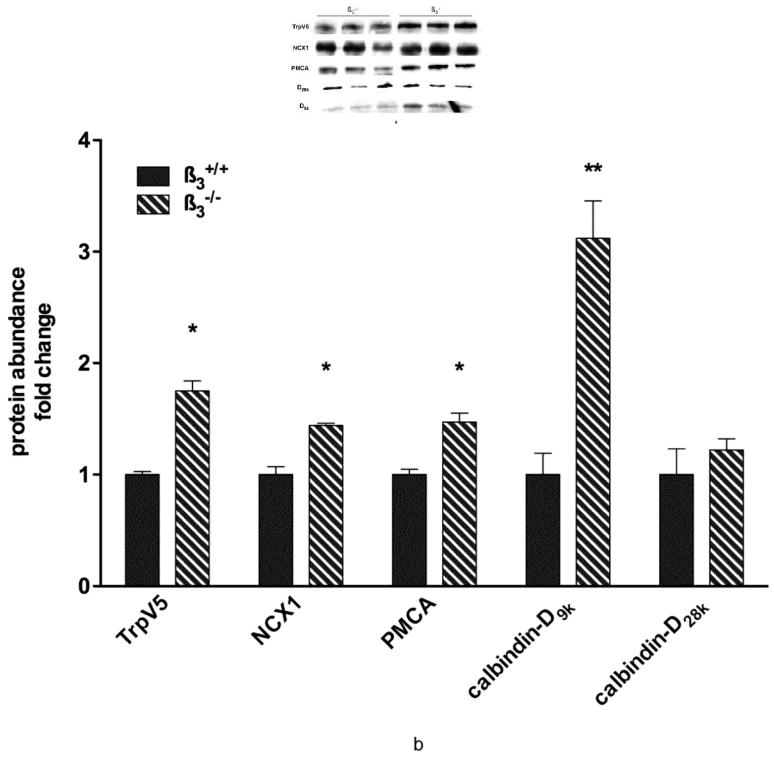

Figure 7.

Calcium transport protein abundance in $Ca_V ß_3^{-/-}$ and wild-type $Ca_V ß_3^{+/+}$ mice. (A) Sample immunoblot of whole kidney homogenates or membranes as described in Materials and Methods. Equal amounts of protein were loaded for each transporter and detected for the indicated protein. (B) Average changes of calcium transporter protein abundance in $Ca_V ß_3^{-/-}$ mice expressed as fold change from wild-type. (*n*=3 for each group). 0.05, ** P < 0.01 *vs.* $Ca_V ß_3^{+/+}$.

Table 1

Comparison of serum electrolytes in conscious wild-type and calcium channel ß$_3$-null mice

	$Ca_V ß_3^{+/+}$	$Ca_V ß_3^{-/-}$
Na, mM	151 ± 7.0	152 ± 4.1
Ca, mM	1.1 ± 0.1	1.3 ± 0.1
K, mM	4.3 ± 0.6	4.4 ± 0.6
n	11	8