Vitamin D Receptor (VDR) Knockout Mice Reveal VDR-Independent Regulation of Intestinal Calcium Absorption and ECaC2 and Calbindin D_{9k} mRNA[1,2]

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ABSTRACT To study the role of calbindin D_{9k} (CaBP) and epithelial calcium channel ECaC2 in intestinal calcium (Ca) absorption, vitamin D receptor knockout (KO) and wild-type (WT) mice were fed either 0.5% Ca or a 2.0% Ca rescue diet starting at 21 d of age. Ca absorption and parameters involved in this process were measured at 60 or 90 d of age. Compared with WT, KO mice fed the 0.5% Ca diet had higher plasma parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], and lower plasma Ca and insulin-like growth factor-I (IGF-I). Duodenal Ca absorption (% Ca absorbed) in KO mice was reduced 71% relative to WT mice and was associated with 55% lower CaBP mRNA, 47% lower CaBP protein and 95% lower ECaC2 mRNA levels. Compared with WT mice, the percentage of Ca absorbed in KO mice fed the 0.5% Ca diet was inappropriately low for the level of duodenal CaBP. The 2% Ca rescue diet normalized plasma Ca, prevented osteomalacia, increased growth and plasma IGF-I levels, but did not normalize plasma PTH or 1,25$(OH)_2D_3$ in KO mice. In addition, the relationship between CaBP protein and the percentage of Ca absorbed was normalized, whereas ECaC2 mRNA fell to near zero. Our data demonstrate that higher CaBP levels do not ensure high rates of duodenal Ca absorption and that transcellular Ca absorption can occur even when ECaC2 gene expression is very low. In addition, our data suggest that the 2% Ca diet promotes a vitamin D receptor–independent anabolic effect on bone formation and calcium absorption, leading to improved calcium balance even in the presence of high PTH levels. J. Nutr. 133: 374–380, 2003.

KEY WORDS: • *calbindin D_{9k}* • *calcium channels* • *calcium absorption* • *bone* • *mice*

Several groups have independently generated vitamin D receptor knockout (VDR KO)[4] mice by targeted disruption of the VDR gene (1–3) or mutant mice expressing VDR without the first zinc finger of the DNA binding domain (4). Like vitamin D–deficient rats, VDR KO and mutant mice are hypocalcemic and exhibit the phenotype of Type II rickets (1). Impaired calcium absorption has been proposed as the primary defect responsible for the phenotype of VDR KO mice (3,5).

Vitamin D–regulated, transcellular calcium absorption is important for maintaining calcium homeostasis (6), especially under the condition of low calcium intake prevalent in U.S. women (7). Defects in calcium absorption can lead to an imbalance in serum calcium levels, resulting in hormonal adaptation that promotes the mobilization of calcium from bone and increasing the risk of osteoporosis (8–10). Three distinct mechanisms have been proposed to explain transcellular calcium absorption, i.e., transcaltachia, a rapid transfer that is likely independent of vitamin D-mediated gene regulation (11), lysosomal transport, a system whereby calcium is sequestered and shuttled through the cell in lysosomes (12–15), and facilitated diffusion, a system that depends upon the presence of the cytosolic calcium binding protein, calbindin D_{9k} (16). In the latter two models, the regulation of calcium absorption by vitamin D is adaptive, occurring over the course of hours (rather than minutes as in transcaltachia) and is presumably dependent upon the presence of the VDR (17).

In the facilitated diffusion model, the central player in the transcellular movement of calcium across the enterocyte is the calcium binding protein, calbindin D_{9k} (16,18,19). Other potentially important proteins in the control of intestinal calcium absorption include the recently identified epithelial calcium channels, ECaC1 (20) and ECaC2 (also called CaT1) (21) and the basolateral calcium pump, PMCA1b (22). Based upon the facilitated diffusion model, the measurable levels of calbindin D_{9k} protein in the VDR KO mice (2;3) should give them the ability to absorb calcium in the intestine through a transcellular, as opposed to paracellular diffusion pathway. Although others have studied calcium absorption in VDR KO

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[4] Abbreviations used: ECaC1, epithelial calcium channel 1; ECaC2, epithelial calcium channel 2 or calcium transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ig, immunoglobulin; IGF-I, insulin-like growth factor-I; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; RT-PCR, reverse transcription-polymerase chain reaction; VDR, vitamin D receptor; VDR KO, vitamin D receptor knockout; WT, wild-type.

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mice (3,5), the effect of the VDR knockout on calcium metabolism has not been fully explored. In this study we have measured the regulation of calcium absorption in the VDR KO mice described by Yoshizawa et al. (1). In addition, we assessed the contribution of renal calcium excretion to the development of the VDR KO phenotype.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified below.

Animals. Two breeding pairs of mice heterozygous for the vitamin D receptor (VDR) knockout (KO) were obtained from Dr. Shigeaki Kato (University of Tokyo, Japan) (1), and fed a commercial rodent diet (8664, Harlan Teklad, Madison, WI). VDR KO and wild-type (WT) mice were generated by breeding heterozygous mice. On d 10 postpartum, tail clips were taken for genotyping (23). On d 21 postpartum, pups were weaned and assigned to the experiments based on their genotype and gender. Throughout the experiments, mice were housed individually and exposed to a 12-h light:dark cycle. Food and water were consumed ad libitum. All of the animal experiments were approved by the Purdue Animal Care and Use Committee.

Experimental design

Experiment I. WT mice were weaned onto the commercial rodent diet (8664, Harlan Teklad, Madison, WI) at 21 d of age. At d 90, they were randomly assigned to one of three AIN-76A–based diets (24) containing either a normal [0.5% calcium (Ca), 0.4% phosphorus (P); normal diet], low (0.02% Ca and 0.35% P) or high calcium content (2.0% Ca, 20% lactose, 1.25% P) (Research Diets, New Brunswick, NJ). All diets contained 0.055 μg vitamin D_3/g. After 7 d of consuming the experimental diets, intestinal calcium absorption from a test dose (2 mmol/L Ca) was determined using the in situ ligated loops procedure described below (n = 10–14 per group). Blood was collected into heparinized tubes by cardiac puncture and plasma was analyzed for 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] levels as described below.

Experiment II. At 21 d of age, male and female WT and VDR KO mice were weaned randomly onto either the normal (0.5%) calcium diet or the high (2%) calcium diet described above. At d 60 (males and females) and 90 (males only), body weight was recorded, and calcium absorption from a 2 mmol/L calcium test dose was determined using the in situ ligated loops procedure described below (n = 5–10 per group). Blood was collected into heparinized tubes by cardiac puncture and plasma was analyzed for Ca, P, parathyroid hormone (PTH), insulin-like growth factor-I (IGF-I) and 1,25$(OH)_2D_3$ levels as described below. The right femur from 60-d-old mice was taken for analysis of calcium content, and urine was collected at the time of the absorption test, either by collecting urine directly into a tube covering the urethra, or by puncturing the bladder with a 25-gauge needle and drawing out the urine with a 1-mL syringe.

A second set of mice (n = 6 per group) was raised under the same protocol and tissue was harvested at 60 d of age for analysis of calbindin D_{9k} protein (duodenum, kidney), calbindin D_{28k} protein (kidney), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), calbindin D_{9k}, ECaC1, ECaC2 (kidney and duodenum) and cdx2 (duodenum) mRNA levels. The first 3 cm of proximal duodenum was excised, rinsed with ice-cold PBS buffer (260 mmol/L NaCl, 10 mmol/L Na_2HPO_4 and 10 mmol/L NaH_2PO_4, pH 7.2), and mucosal scraping was obtained; 20% of the scraping was placed in 1 mL of Tri-Reagent (Molecular Research, Cincinnati, OH), vortexed to disrupt the sample and saved at −80°C for later analysis. The right kidney was removed and minced. One half of the minced kidney was placed in 1 mL of Tri-Reagent, and saved at −80°C for later analysis. The remainder of duodenal scraping and kidney was flash frozen in liquid nitrogen and stored at −80°C for later analysis of specific protein levels. Before tissue harvest, blood was collected into heparinized tubes by cardiac puncture and plasma was analyzed for Ca, P, PTH, IGF-I and 1,25$(OH)_2D_3$ levels as described below.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using Tri-Reagent as per the manufacture's directions (Molecular Research Center, Cincinnati, OH). Calbindin D_{9k}, ECaC1, ECaC2, cdx-2, and GAPDH mRNA levels were assessed by semiquantitative RT-PCR as previously described (25). Primers and annealing temperatures are: calbindin D_{9k}, forward: ATGTGTGCTGAGAAGTCTCCTGCAGAAATG, reverse: CATTGTGAGAGCTTTTTGAAGAAAGCTTCG, annealing temperature (Ta) = 59°C, GenBank ID = AF136283; ECaC1, forward: CGTTGGTTCTTACGGGTTGAAC, reverse: GTTTGGAGAACCACAGAGCCTCTA, Ta = 55°C, GenBank ID = AF336378; ECaC2, forward: CAAGATCTCAACAGACAACGC, reverse: TAGATCTGGTACTCCCAGCCCTC, Ta = 55°C, GenBank ID = AB037373; Cdx2, forward: CCCAGCGGCCAGCGGCGAAACCTGT, reverse: TTCTCGCAGCGTCCATACTCCTCAT, Ta = 65°C, GenBank ID = U00454; GAPDH, forward: CCATGGAGAAGGCTGGGG, reverse: CAAAGTTGTCATGGATGACC, Ta = 55°C, GenBank ID = X02231.

PCR cycle numbers used were: calbindin D_{9k} (duodenum and kidney: 20 cycles), ECaC1 (duodenum: 35 cycles; kidney: 32 cycles), ECaC2 (duodenum: 25 cycles for WT mice, and 30 cycles for VDR KO mice; kidney: 31 cycles), cdx2 (28 cycles) and GAPDH (18 cycles). These cycles were chosen so that the amplification was conducted in the linear range of amplification efficiency for each of the primer sets (data not shown). The resulting PCR products were subjected to electrophoresis on 2.5% agarose gels containing ethidium bromide and bands were visualized under UV light. Gel data were recorded using the BioRad FluorS Imaging System and relative densities of the bands were determined using Quantity One software (BioRad, Hercules, CA). Data were normalized for the expression of GAPDH within the sample. For ECaC2 and ECaC1, we adjusted the data to account for differences in cycle numbers using the equation $An = Ao(1 + R)^n$ to determine a correction factor where An is the amount of product produced at n cycles, Ao is the starting amount of cDNA, R is the amplification efficiency (assumed to be 0.8) and n is the cycle number. Using this correction factor permitted us to compare the transcript levels for ECaC2 and ECaC1 within and between tissues.

Western blot analysis of calbindin D_{9k} and calbindin D_{28k}. *Sample preparation.* Duodenal scrapings or kidneys were homogenized 10:1 (vol/wt) in ice-cold homogenization buffer (10 mmol/L Tris-HCl, 5 mmol/L benzamidine and 1 mmol/L β-mercaptoethanol, along with 230 trypsin inhibitor units (TIU)/L aprotinin, 0.05 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 complete protease inhibitor tablet per 50 mL of buffer, pH 7.2). The homogenates were centrifuged at 100,000 × g at 4°C for 1 h. The resulting supernatant (cytosol) was used for analysis of calbindin D_{9k} and calbindin D_{28k} protein by Western blotting.

Western blot procedure. Western blot analysis was conducted as previously described (26). Cytosolic protein (5 μg) was subjected to SDS-PAGE on a 16% Tris-glycine gel (NOVEX, San Diego, CA). After the electrophoresis, the proteins were transferred electrophoretically to Immobilon-P membrane (0.1-μm pore size, Millipore, Bedford, MA), following the manufacturer's instructions. The detection procedure was carried out at room temperature using 1:1000 dilutions of either rabbit anti-rat calbindin D_{9k} antibody (SWant, Bellinzona, Switzerland) or a mouse anti-chicken calbindin D_{28k} antibody (SWant) for the primary antibody and 1:5000 dilutions of horseradish peroxidase–linked donkey anti-rabbit immunoglobulin (Ig)G for calbindin D_{9k} or donkey anti-mouse IgG for calbindin D_{28k} for the secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Specific binding was detected using the ECL$^+$ detection reagents (Amersham Life Sciences, Arlington Heights, IL) according to manufacture's directions. The chemiluminescent signal was visualized and recorded using the FluorS Imaging system. The relative band densities were quantified using Quantity One software (Bio-Rad).

Calcium absorption. The capacity of the duodenum to absorb calcium was assessed after adapting mice to different diets using a modification of the in situ ligated loop of duodenum procedure described by others (27,28). Because calbindin D_{9k} mRNA is expressed primarily in the proximal small intestine in mice (29), the

calcium absorption test was performed in the duodenum. Mice were deprived of food for 12 h before the test. ^{45}Ca buffer [40 μL; 2 mmol/L $CaCl_2$, 150 mmol/L NaCl, 30 mmol/L Tris and 0.592 MBq/L ^{45}Ca (Amersham), pH 7.2] was injected into the lumen through the tightened distal ligature. At this concentration of calcium, active calcium transport predominates (16,28). After 10 min of incubation, the loop was removed and digested in 2 mL of Soluene-350 tissue solubilizer (Packard, Meriden, CT) at 65°C overnight. Aliquots of the digested intestinal segment were analyzed using a liquid scintillation counter. The efficiency of calcium absorption during the 10-min incubation period was determined by measuring the disappearance of ^{45}Ca from the loop, i.e., [1 − (amount of Ca remaining in the loop/amount of Ca injected into the loop)] · 100.

Urinary calcium and creatinine analysis. Samples were stored at –20°C and sent to the Nutrition Evaluation Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University for analysis. Urinary calcium was determined by direct current plasma emission spectroscopy (Beckman Instruments, Palo Alto, CA) and creatinine was measured using a Cobas Fara centrifugal analyzer (Roche Instruments, Belleville, NJ).

Biochemical assays. *Plasma analysis.* Plasma $1,25(OH)_2D_3$, PTH and IGF-I levels were determined by a ^{125}I-$1,25(OH)_2D_3$ RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), a mouse intact PTH ELISA kit (Immutopics, San Clemente, CA) and a rat ^{125}I-IGF-I RIA kit (Diagnostic System Laboratories, Webster, TX) following the manufactures' directions. Plasma was diluted with 2% nitric acid, and calcium and phosphorus contents were measured using Inductively Coupled Plasma-Optical Emission Spectrometry (Perkin Elmer, Norwalk, CT).

Femoral analysis. At the end of each experiment, the right femur was excised and all tissue was scraped from the bone using a scalpel. Femur length was measured using calipers, the femur was dried at 50°C for 48 h and dry weight was recorded. Bones were ashed for 6 h at 300°C followed by 24 h at 600°C and the ash weight was recorded. Ash was dissolved in 1 mol/L HCl and then further diluted in 0.5 mol/L HCl containing 0.5% lanthanum chloride. Calcium content was measured by atomic absorption spectrometry (Perkin Elmer Instruments, Norwalk, CT), and expressed as mg/g dry weight.

Statistical analysis. Data were analyzed using the SAS statistical program (version 8.0, SAS Institute, Cary, NC). When the plots of predicted values vs. residuals demonstrated that the data were not normally distributed, log or square root transformations were conducted before statistical analysis. Main effects and interactions between factors (genotype, diet) were analyzed by the General Linear Models procedure. Gender was used as a covariate. For calcium absorption studies, the results were not influenced by loop length (i.e., significance levels were not altered by using loop length as a covariate). Linear regression analysis was applied to determine the correlation between calbindin D_{9k} protein and mRNA in 2-mo-old mice, and between intestinal calcium absorption and calbindin D_{9k} protein in 2- and 3-mo-old mice. Comparisons of multiple group means were done using Fisher's Protected Least Significant Difference test. Differences were considered statistically significant when $P < 0.05$. Data are expressed as means ± SEM.

RESULTS

Body weight and femur parameters. VDR KO mice fed a normal calcium diet until 60 d of age were significantly lighter than WT mice fed the same diet (**Table 1**). The adverse effect of the 0.5% calcium diet was particularly prominent for males at 90 d of age. Although WT males reached a mature body weight by 60 d (27.6 g), VDR KO growth was 33% less at this time. The mice did not gain any additional weight over the next month (data not shown). The body weights of VDR KO mice fed the high calcium rescue diet were not significantly different from those of WT mice fed this diet at either 60 (Table 1) or 90 d (data not shown). Femur length, femur dry weight and percentage ash weight were significantly decreased in 60-d-old VDR KO mice fed the normal diet compared with WT mice (15, 35 and 18% lower, respectively). Femoral calcium content was reduced by 22% in the VDR KO mice fed the normal diet (Table 1). The high calcium diet significantly reduced these effects on femur length, dry weight, percentage ash weight, and calcium content in VDR KO mice (11, 34, 16 and 22% higher relative to VDR KO fed the normal diet, respectively).

Plasma parameters. VDR KO mice fed the normal calcium diet were hypocalcemic at 60 d of age (plasma calcium levels were 48% lower than WT mice, Table 1). As a result, plasma PTH and $1,25(OH)_2D_3$ levels were 34- and 18-fold higher, respectively, than WT in 60-d-old mice. IGF-I levels were 29% lower in VDR KO mice than WT mice fed the normal diet at 60 d of age. The high calcium diet significantly reduced the plasma PTH and $1,25(OH)_2D_3$ levels by 50 and

TABLE 1

Serum variables, bone, urinary calcium excretion and body weight in 60-d-old WT and KO mice fed either a 0.5 or 2.0% Ca diet

	WT		KO	
	0.5% Ca	2.0% Ca	0.5% Ca	2.0% Ca
Plasma				
Ca, *mmol/L*	1.74 ± 0.04^{c} (12)	1.62 ± 0.04^{b} (12)	0.90 ± 0.02^{a} (21)	1.60 ± 0.04^{b} (21)
P, *mmol/L*	4.75 ± 0.02^{a} (12)	4.40 ± 0.19^{a} (12)	4.45 ± 0.13^{a} (21)	4.45 ± 0.13^{a} (21)
PTH, *pmol/L*	5.2 ± 0.9^{b} (12)	2.6 ± 0.5^{a} (12)	182.2 ± 19.5^{c} (12)	153.8 ± 20.1^{c} (12)
$1,25(OH)_2D_3$, *pmol/L*	380 ± 60^{b} (6)	85 ± 12^{a} (6)	7705 ± 860^{c} (6)	8430 ± 980^{c} (6)
IGF-I, *nmol/L*	63.2 ± 2.1^{b} (11)	62.7 ± 2.3^{b} (11)	45.0 ± 2.5^{a} (11)	58.6 ± 2.5^{b} (11)
Bone				
Femur length, *mm*	14.3 ± 0.1^{c} (12)	14.3 ± 0.1^{c} (12)	12.1 ± 0.1^{a} (21)	13.4 ± 0.1^{b} (21)
Dry weight, *mg*	32.7 ± 1.0^{c} (12)	35.3 ± 1.0^{c} (12)	21.4 ± 0.8^{a} (21)	28.6 ± 0.7^{b} (21)
Ash weight, *%*	57.2 ± 0.6^{c} (12)	58.1 ± 0.7^{c} (12)	46.8 ± 1.1^{a} (21)	54.2 ± 0.9^{b} (21)
Femoral Ca, *mg/g of dry weight*	194.1 ± 2.0^{c} (12)	197.4 ± 2.1^{c} (12)	150.9 ± 2.9^{a} (21)	183.4 ± 5.2^{b} (21)
Urine, *Ca/Creatinine ratio*	0.07 ± 0.01^{a} (12)	0.50 ± 0.1^{c} (12)	0.18 ± 0.04^{b} (21)	0.10 ± 0.02^{b} (21)
Body weight, *g*				
Male	27.6 ± 2.7^{c} (5)	25.2 ± 0.8^{bc} (6)	18.6 ± 0.6^{a} (10)	22.7 ± 1.2^{b} (8)
Female	20.4 ± 1.0^{b} (7)	21.3 ± 1.7^{b} (6)	16.8 ± 0.7^{a} (11)	18.6 ± 0.6^{ab} (13)

1 Ca, calcium; P, phosphorus; IGF-I, insulin-like growth factor-I; $1,25(OH)_2D_3$, 1, 25 dihydroxyvitamin D_3; PTH, parathyroid hormone; KO, vitamin D receptor knockout; WT, wild-type.

2 Values are means ± SEM, (*n*); values with different superscript letters in a row differ, $P < 0.05$.

75%, respectively, in WT mice. In VDR KO mice, the high calcium diet normalized plasma calcium but had no effect on plasma PTH or $1,25(OH)_2D_3$ (Table 1). Plasma IGF-I levels were normalized in VDR KO mice fed the high calcium diet.

Intestinal calcium absorption. Intestinal calcium absorption from a test dose was 17.3 ± 2.0% in 3-mo-old WT mice fed the 0.5% calcium diet. Feeding a 2% calcium diet for 1 wk significantly suppressed calcium absorption by 75% (4.4 ± 0.5%), whereas feeding a 0.02% calcium diet for 1 wk increased calcium absorption 2.3-fold compared with the 0.5% calcium diet (57.2 ± 2.8%). This was associated with similar changes in plasma $1,25(OH)_2D_3$ (0.02% calcium diet, 556 ± 29 pmol/L; 0.5% calcium diet, 319 ± 36 pmol/L; 2% calcium diet, 58 ± 12 pmol/L).

In the experiment comparing intestinal calcium absorption in VDR KO and WT mice fed either a 0.5% calcium or 2% calcium diet from weaning, we observed a significant genotype × diet interaction ($P < 0.0001$) (**Table 2**). Compared with WT mice, calcium absorption in VDR KO mice fed the normal diet was reduced by 71% in 2-mo-old mice and by 87% in 3-mo-old mice. In the 3-mo-old male VDR KO mice, calcium absorption was equal to the calculated rate of paracellular diffusion [2.3 ± 1.1%/10 min or 13.8%/h (28)]. Feeding the high calcium diet to the WT group caused a 64% reduction in the efficiency of intestinal calcium absorption in 2-mo-old mice and 65% reduction in 3-mo-old mice (Table 2). In contrast, compared with VDR KO mice fed the 0.5% calcium diet, calcium absorption efficiency of the intestine was increased by 13% in 2-mo-old VDR KO ($P = 0.12$) and by 335% in 3-mo-old male VDR KO ($P < 0.01$) fed the high calcium diet.

Duodenal protein and gene expression. In 2-mo-old mice, calbindin D_{9k} protein (**Fig. 1**) and mRNA were 55 and 47% lower in VDR KO relative to WT mice fed the 0.5% calcium diet (**Table 3**). The high calcium diet significantly reduced calbindin D_{9k} protein and mRNA levels in WT mice by 70 and 71%, respectively; this effect was present but reduced in VDR KO mice (52 and 33% reduction in protein and mRNA, respectively). Similar changes were also seen for calbindin D_{9k} protein levels in 3-mo-old mice (0.5% Ca diet, calbindin D_{9k} protein in VDR KO was 28% lower than WT; 2% Ca diet suppressed calbindin D_{9k} protein levels by 78% in WT and 28% in VDR KO mice) (data not shown). The correlation between calbindin D_{9k} protein and mRNA levels was strong (in 2-mo-old mice, calbindin D_{9k} protein = 0.135(calbindin D_{9k} mRNA) – 0.02, $r^2 = 0.87$, $P < 0.05$).

A regression analysis between calbindin D_{9k} protein and intestinal calcium absorption was conducted in 60- and 90-d-old mice (**Fig. 2**). Data are presented as group means and were expressed relative to the value for the 2-mo-old WT group fed the normal diet (2 mo, WT, 0.5% Ca = 100). Calcium absorption was closely related to intestinal calbindin D_{9k} protein levels in WT mice fed either diet and in VDR KO mice fed the 2% Ca diet [calcium absorption = 0.94(calbindin D_{9k} protein) + 5.7, $r^2 = 0.98$, $P < 0.05$]. The data from the VDR KO groups fed the 0.5% Ca diet fell outside the 95% confidence interval of the regression line of this relationship.

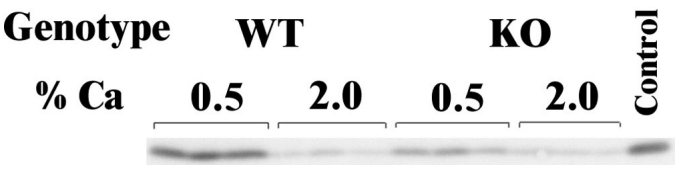

FIGURE 1 Western blot of duodenal calbindin D_{9k} protein levels in 2-mo-old wild-type (WT) or vitamin D receptor knockout (KO) mice fed a 0.5% or 2% calcium (Ca) diet. Bovine calbindin D_{9k} protein was used as a control. Data are presented for three individual mice per group.

ECaC1 mRNA levels were <1% of ECaC2 mRNA levels in WT mice (Table 3). Both ECaC1 and ECaC2 mRNA levels were lower in VDR KO mice than in WT mice fed the 0.5% Ca diet (by 74 and 95%, respectively). The high calcium diet reduced ECaC1 and ECaC2 mRNA levels equally in WT and VDR KO mice (by 39–53% for ECaC1 and 74–81% for ECaC2). Duodenal cdx2 mRNA levels were expressed in WT and KO mice fed either diet, but neither genotype nor diet influenced cdx2 mRNA levels (Table 3).

Urinary calcium excretion. VDR KO mice had significantly higher calcium excretion (1.6-fold higher, $P < 0.05$) compared with WT mice fed the normal calcium diet (Table 1). Feeding a high calcium diet to WT mice caused a 6.3-fold increase in the urinary calcium to creatinine ratio. This effect was not observed in VDR KO mice.

Renal protein and gene expression. There was no detectable renal calbindin D_{9k} protein in VDR KO mice in either diet group, and the high calcium diet did not alter renal calbindin D_{9k} protein levels in WT (Table 3). Similarly, renal calbindin D_{9k} mRNA levels were significantly reduced in VDR KO mice compared with WT (96% reduction in KO fed the normal diet, 84% the high calcium diet) and were not significantly affected by dietary calcium level in WT or VDR KO mice. Calbindin D_{28k} protein levels were significantly reduced (59%) in VDR KO mice fed the normal diet compared with WT. The high calcium diet did not affect the level of this protein in WT mice, but calbindin D_{28k} protein was doubled by the high calcium diet in VDR KO mice. VDR KO mice had higher renal ECaC1 and ECaC2 mRNA levels than WT mice but diet had no effect on these mRNA levels in either WT or VDR KO mice (Table 3).

TABLE 2

Calcium absorption in 60 and 90-day-old WT and KO mice fed either a 0.5 or 2.0% Ca diet

	WT		KO	
	0.5% Ca	2.0% Ca	0.5% Ca	2.0% Ca
		%		
60-day old	55.4 ± 3.8[c] (11)	20.0 ± 1.7[b] (10)	16.3 ± 1.6[a] (18)	18.4 ± 1.5[ab] (14)
90-day old	18.0 ± 0.9[d] (4)	6.3 ± 0.5[b] (5)	2.3 ± 1.1[a] (4)	10.0 ± 1.4[c] (4)
60-d/90-d Ratio	3.1	3.2	7.1	1.8

[1] Ca, calcium; KO, vitamin D receptor knockout; WT, wild-type.
[2] Values are mean ± SEM, (*n*); values with different superscript letters in a row differ, $P < 0.05$.

TABLE 3

mRNA and protein levels in the duodenum and kidney of 60-d-old WT and KO mice fed either a 0.5 or 2.0% Ca diet[1,2]

		WT		KO	
		0.5% Ca	2.0% Ca	0.5% Ca	2.0% Ca
	n	*arbitrary units*			
mRNA					
Duodenum	6				
Calbindin D_{9k}		5.1 ± 0.7^{b}	1.5 ± 0.2^{a}	2.7 ± 0.6^{b}	1.8 ± 0.2^{a}
ECaC1		6 ± 2^{c}	4 ± 1^{bc}	2 ± 1^{ab}	0.7 ± 0.1^{a}
ECaC2		1560 ± 408^{c}	410 ± 119^{b}	79 ± 32^{a}	15 ± 4^{a}
Cdx2		0.8 ± 0.1^{a}	1.2 ± 0.3^{ab}	1.4 ± 0.4^{cb}	1.2 ± 0.4^{ab}
Kidney	10				
Calbindin D_{9k}		1.6 ± 0.7^{b}	0.5 ± 0.1^{b}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}
ECaC1		48 ± 5^{a}	44 ± 4^{a}	65 ± 7^{b}	66 ± 12^{b}
ECaC2		10 ± 2^{a}	7 ± 1^{a}	18 ± 3^{b}	16 ± 2^{b}
Protein					
Duodenum	6				
Calbindin D_{9k}		6.9 ± 0.4^{c}	2.1 ± 0.6^{a}	3.1 ± 0.7^{b}	1.5 ± 0.3^{a}
Kidney	10				
Calbindin D_{9k}		2.0 ± 0.2^{a}	2.0 ± 0.3^{a}	ND	ND
Calbindin D_{28k}		3.2 ± 0.5^{b}	2.5 ± 0.3^{b}	1.3 ± 0.1^{a}	2.8 ± 0.3^{b}

[1] Ca, calcium; ECaC1, epithelial calcium channel 1; ECaC2, epithelial calcium channel 2 or calcium transporter 1; GAPDH, glyceraldehydes phosphate dehydrogenase; ND, non-detectable; KO, vitamin D receptor knockout; WT, wild-type.

[2] Data are expressed as mean ± SEM. Values with different superscript letters in a row differ, $P < 0.05$.

DISCUSSION

Several groups have previously studied the effect of VDR gene ablation or mutation on calcium and bone metabolism using mice generated in Boston, MA (2,5,30,31), Tokyo, Japan (1,32), Leuwen, Belgium (3) and Munich, Germany (4). Our data from the Tokyo VDR knockout mouse confirm the data from these studies, i.e., we showed that the growth suppression and impaired bone mineralization associated with the VDR knockout could be limited by feeding a 2% calcium, 20% lactose diet. However, our data add to these previous reports in several critical ways.

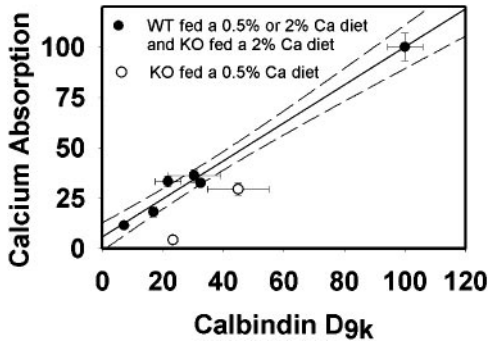

FIGURE 2 The correlation between intestinal calcium (Ca) absorption and calbindin D_{9k} protein levels in wild-type (WT) and vitamin D receptor knockout (KO) mice fed a 0.5% or 2% Ca diet. Group means for the percentage of calcium absorption and calbindin D_{9k} protein from 60- and 90-d-old WT and KO mice were expressed relative to the levels found in 60-d-old WT mice fed the 0.5% Ca diet (2 mo WT, 0.5% Ca = 100). The solid line represents the linear regression between calcium absorption and intestinal calbindin D_{9k} levels in all groups except the KO mice fed the 0.5% Ca diet (calcium absorption = 0.94(calbindin D_{9k} protein) + 5.7, $r^2 = 0.98$) ($P < 0.05$); the dashed line is the 95% confidence interval around the regression line.

Van Cromphaut et al. (3) and Li et al. (5) reported previously that the primary defect contributing to the VDR KO mouse phenotype is impaired calcium absorption. Like them, we found that calcium absorption was reduced by 71% in 60-d-old VDR KO mice fed a normal calcium diet compared with WT (Table 2). Intestinal calcium absorption is a vitamin D–regulated process and, as others have shown in vitamin D–deficient rats (33–35), we expected to observe only non-regulated, diffusional transport in VDR KO mice [transport <16%/h in mice and rats (28;36)]. However, using an in situ loop procedure and a low calcium load to follow the transcellular portion of calcium absorption (28), we found that absorption of calcium from a test dose in VDR KO mice was at least 2.7-fold higher than paracelluar calcium transport in every group we studied, with the exception of the severely growth arrested, 3-mo-old males fed the 0.5% calcium diet (2.3% transported in 10 min or 13.8%/hour, i.e., equal to diffusion). Surprisingly, in 3-mo-old mice, calcium absorption efficiency in Tokyo VDR KO mice fed the rescue diet was 3.3-fold higher than in VDR KO mice fed the 0.5% calcium diet; a similar effect was also seen in 2-mo-old VDR KO mice. We do not believe that this reflects an increase in the paracellular diffusion in VDR KO mice fed the rescue diet; cell culture studies suggest that the absence of calcium resulting from EGTA treatment leads to increased paracellular diffusion between enterocytes (37). These data suggest that a constraint imposed upon transcellular intestinal calcium absorption in VDR KO mice fed the normal calcium diet was removed by the rescue diet.

By using a controlled absorption test in the duodenum and determining the level of calbindin D_{9k} protein and ECaC1 and -2 mRNAs in that same segment, we are able to assess more completely the role of these proteins in calcium absorption. The relationship between calcium absorption efficiency and intestinal calbindin D_{9k} content is normally a tight linear relationship (16,33). However, in VDR KO mice fed the 0.5%

calcium diet, the level of duodenal calcium absorption was lower than one would predict based upon the calbindin D_{9k} content (Fig. 2), i.e., high calbindin D_{9k} levels alone are not sufficient to drive intestinal calcium absorption in the VDR KO, as the facilitated diffusion model proposes (16). This observation is consistent with several other studies in animals (38;39) and our recent data from Caco-2 cell clones (26). However, although our observation raises doubts about the role of calbindin D_{9k} as a ferry protein whose cellular level is the rate-limiting determinant of intestinal calcium absorption, they do not eliminate the possibility that this protein is important for the process as an intracellular calcium buffer. The precise role of calbindin D_{9k} in calcium absorption awaits the production of a calbindin D_{9k} knockout mouse.

The recent discovery of two brush border calcium channels, ECaC1 (20) and ECaC2 (21), increased interest in the process of calcium uptake into enterocytes during absorption. Like others (3;5), we found that ECaC2 mRNA was much higher than ECaC1 in the duodenum, thus suggesting a greater role for ECaC2 in the duodenum. Previously, we showed that ECaC1 mRNA levels were essentially undetectable in Caco-2 cells and that the relative induction of ECaC2 mRNA by $1,25(OH)_2D_3$ correlates well with vitamin D–induced calcium transport in Caco-2 clones (26). Similarly, the parallel changes in calcium absorption efficiency and ECaC2 mRNA levels noted upon changing dietary calcium levels in WT mice are consistent with an essential role for this protein in vitamin D–induced calcium absorption and the mathematical model of transcellular calcium transport reported by Slepchenko and Bronner (40). However, our findings in VDR KO mice fed the high calcium diet do not appear to fit this model. Calcium absorption efficiency increased by 13% in 2-mo-old VDR KO mice fed the high calcium diet even though the expression of ECaC2 mRNA was <1% of the level seen in WT mice fed the 0.5% calcium diet. Previously, Chandra et al. (41) showed that calcium ions could enter the enterocyte at the brush border membrane even in vitamin D–deficient chickens. This suggests that calcium uptake is not a significant limitation to calcium absorption in the absence of vitamin D. Thus, these low levels of message may produce enough protein to permit calcium uptake at the brush border of the enterocyte. Future studies measuring ECaC2 protein levels in the duodenum of the VDR KO mouse and the assessment of calcium uptake into brush border membrane vesicles under various conditions will be necessary to clarify the role of ECaC2 in intestinal calcium absorption.

Another interesting finding from our work relates to the regulation of ECaC and calbindin D_{9k} levels by $1,25(OH)_2D_3$. Like Van Cromphaut et al. (3), we found that the high calcium rescue diet reduced duodenal ECaC2 mRNA, ECaC1 mRNA and calbindin D_{9k} mRNA levels significantly in WT mice, and these changes were associated with a 75% decline in plasma $1,25(OH)_2D_3$ levels. ECaC2 mRNA levels are strongly up-regulated by $1,25(OH)_2D_3$ treatment in Caco-2 cells (26,42) and the response of intestinal calbindin D_{9k} to $1,25(OH)_2D_3$ treatment has been well documented by others (19). This suggests that the suppression of these parameters by the high calcium rescue diet in WT mice is through the loss of vitamin D--dependent, nuclear VDR signaling. However, the rescue diet had the same effect on ECaC1, ECaC2 (mRNA) and calbindin D_{9k} (mRNA and protein) in VDR KO mice. This demonstrates that the effect of a high calcium diet on these parameters, although coincident with a drop in serum $1,25(OH)_2D_3$ in WT mice, does not require the nuclear VDR signaling pathway. In addition, because these changes were observed in our study without alteration in plasma $1,25(OH)_2D_3$ by the rescue diet, we believe that it is also unlikely that the nongenomic vitamin D signaling pathway (43) is involved. Further study is required to determine the mechanism of down-regulation of these messages by the high calcium diet.

Our data show that the improvements in bone and calcium metabolism in VDR KO mice fed the high calcium diet are not simply due to correction of elevated plasma PTH levels (Table 1). One potential contributor to this effect may be IGF-I, a growth factor that mediates the action of growth hormone during linear growth (44). IGF-I has been identified as an important regulator of bone formation (45,46) and intestinal calcium absorption (47,48). We observed that the 0.5% calcium diet significantly suppressed the growth of VDR KO mice and that this was associated with a 29% reduction in serum IGF-I levels. The high calcium diet improved both plasma IGF-I levels and body weight in VDR KO mice. We showed previously that growth hormone causes a vitamin D-independent increase in intestinal calcium absorption in aged male rats (47) and stimulates BMP-2–induced bone formation, coincident with a 50% increase in serum IGF-I levels (49). Although others have suggested that the effect of growth hormone and IGF-I on calcium metabolism is through renal production of $1,25(OH)_2D_3$ (50), our studies suggest that the effect of IGF-I on calcium absorption is independent of the vitamin D axis. Further studies will be necessary to determine the role of IGF-I in calcium absorption during growth.

In conclusion, we have conducted a detailed examination of calcium metabolism of the Tokyo VDR KO mouse, which has revealed several important issues related to intestinal calcium absorption and the regulation of genes/proteins involved in this process. First, the release of growth inhibition in VDR KO mice fed a rescue diet leads to a vitamin D receptor–independent modulation of calcium absorption and bone formation perhaps through an IGF-I dependent mechanism. Second, the suppression of ECaC1, ECaC2 and calbindin D_{9k} mRNA, and calbindin D_{9k} protein levels in the duodenum of mice fed a high calcium diet is not mediated by changes in plasma $1,25(OH)_2D_3$ levels or through a nuclear VDR-mediated mechanism, i.e., they occur even in VDR KO mice. Finally, high levels of calbindin D_{9k} alone are not sufficient to drive high rates of intestinal calcium absorption, but calcium absorption can increase even when ECaC1 and ECaC2 mRNA levels are very low in the duodenum. This study demonstrates that vitamin D is not the only factor capable of regulating calcium absorption or the proteins thought to be involved in the process, and it identifies several potential mechanisms for regulation that should be investigated in the future.

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