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Intestinal Calcium Absorption: Molecular Vitamin D Mediated Mechanisms

R. Bouillon,* S. Van Cromphaut, and G. Carmeliet

Laboratorium voor Experimentele Geneeskunde en Endocrinologie (LEGENDO), Onderwijs en Navorsing, Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

Abstract Rickets and hyperparathyroidism caused by a defective Vitamin D receptor (VDR) can be prevented in humans and animals by high calcium intake, suggesting that intestinal calcium absorption is critical for $1,25(OH)_2$ vitamin D [$1,25\text{-}(OH)_2D_3$] action on calcium homeostasis. We assessed the rate of serum ^{45}Ca accumulation within 10 min after oral gavage in two strains of VDR-knock out (KO) mice (Leuven and Tokyo KO) and observed a threefold lower area under the curve in both KO-strains. Moreover, we evaluated the expression of intestinal candidate genes, belonging to a new class of calcium channels (TRPV), involved in transcellular calcium transport. The calcium transport protein ECaC2 was more abundantly expressed at mRNA level than ECaC1 in duodenum, but both were considerably reduced (ECaC2 > 90%, ECaC1 > 60%) in the two VDR-KO strains on a normal calcium diet. Calbindin-D_{9K} expression was only significantly decreased in the Tokyo KO, whereas $PMCA_{1b}$ expression was normal in both VDR-KOs. In Leuven wild type mice, a high calcium diet inhibited (> 90%), and $1,25(OH)_2D_3$ or low calcium diet induced (sixfold) duodenal ECaC2 expression and, to a lesser degree, ECaC1 and calbindin-D_{9K} expression. In Leuven KO mice, however, high or low calcium intake decreased calbindin-D_{9K} and $PMCA_{1b}$ expression, whereas both ECaC mRNA expressions remained consistently low on any diet. These results suggest that the expression of the novel duodenal epithelial calcium channels (in particular ECaC2 or TRPV6) is strongly vitamin D dependent and that calcium influx, probably interacting with calbindin-D_{9K}, should be considered as a rate-limiting step in the process of vitamin D dependent active calcium absorption. J. Cell. Biochem. 88: 332–339, 2003.

Key words: vitamin D; TRP channels; calbindins; epithelial calcium channel; ATPase; vitamin D receptor

Intestinal calcium absorption determines the availability of calcium for different functions in the body. Intracellular calcium is of vital importance for numerous cellular functions and extracellular calcium is equally important for the regulation of muscle and nerve function, blood coagulation as well as for development and maintenance of bone and teeth structures. Calcium transport across cell membranes (intracellular as well as plasma membranes) is thus universal and numerous calcium transporters are therefore highly specialised and regulated proteins belonging to different subclasses of proteins.

The external calcium balance depends on the net transport of calcium across the intestine and the regulated loss of calcium via the kidney and less controllable cutaneous losses. During reproduction, additional important calcium transport and net losses for the mother (and gain for the foetus/neonate) occur across the placenta and breast. Net intestinal calcium transport is determined by the availability of this ion in the diet, its solubility in the gut, and the net capacity to absorb it across the intestine. In general, net calcium absorption is the balance of gross calcium absorption (via a saturable transcellular and a non-saturable paracellular route) and calcium secretion in the gut via different organs (gastric, biliary, pancreatic and intestinal secretions). In the kidney, calcium is massively filtered (up to 10 g/day) and then equally massively reabsorbed in the proximal convoluted tubule with fine-tuning of the net urinary losses by selective calcium re-absorption in the distal part of the nephron via molecular mechanisms resembling the intestinal active calcium

*Correspondence to: R. Bouillon, LEGENDO, Onderwijs en Navorsing, Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. E-mail: Roger.Bouillon@med.kuleuven.ac.be

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absorption. In the intestine, on the contrary, dietary calcium first meets the most active and regulated regions proximally (duodenum and proximal jejunum) whereas more distally less saturable and more non-saturable calcium absorption occurs.

The intestinal calcium absorption depends on many factors both exogenous (dietary intake of calcium and other nutrients such as phosphate or phytates . . .) and endogenous such as gastric acid secretion but especially the presence of active vitamin D. Other hormones such as parathyroid hormone (PTH), glucocorticoids, estrogens, or other pregnancy related factors, growth hormone and insulin like growth factor (GH/IGF) may also influence calcium transport but it is presently unclear to what extend their effect is direct or totally vitamin D mediated. Whereas the molecular mechanisms involved in paracellular calcium transport are totally unclear unless related to the function of tight junctions, the transcellular epithelial/intestinal calcium transport is a multi-step process consisting of at least three phases: (1) calcium entry across the brush border initially thought to be passive, (2) the intracellular calcium transport and (3) finally the calcium extrusion into the blood stream at the serosal site (Fig. 1). Whereas it is well known that the active vitamin D hormone $1,25\text{-}(OH)_2D$ is essential for this transport, the molecular mechanisms involved were less well understood.

Vitamin D resistant rickets due to the absence of a functional VDR is an autosomal recessive disorder, which creates target tissue resistance to the actions of $1,25\text{-}(OH)_2D$. The severe rickets of such children can be remarkably well cured by intravenous calcium infusions [Balsan et al., 1986] or by long-term treatment with high oral doses of calcium [Hochberg et al., 1992]. The bone abnormalities due to severe simple vitamin D deficiency can also be (nearly) normalized by prolonged calcium infusion [Weinstein et al., 1984]. These observations have been confirmed in animal models of vitamin D resistance as the bone

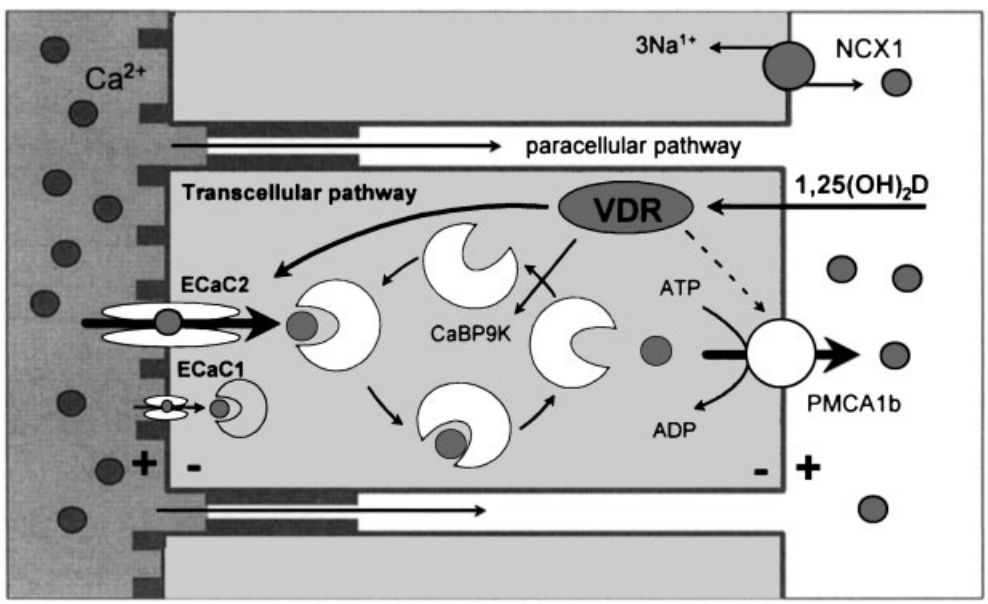

Fig. 1. Molecular model for intestinal calcium absorption. Paracellular calcium absorption is largely a passive mechanism driven by luminal calcium concentration and the integrity of intercellular tight junction. The transcellular pathway involves the epithelial calcium entry through specialised calcium channels (ECaC2 and to a much lesser extent ECaC1). These channels seem to be constitutively in the ''open'' modulus and influx can therefore be regulated by the number of channels. These channels, however, are highly dependent on intracellular free calcium concentration and to remain in the ''open'' state requires the constant buffering of entering calcium by intracellular calcium binding proteins. Calbindin-D_{9K} is the major intracellular calcium binding protein in the mammalian intestinal cell but the contribution of other proteins (CaBP-D_{28K}, calmodulin, etc.) is unknown. The molecular mechanism involved in the transfer of calcium between the different proteins is also not fully explored (direct protein–protein interaction or other helper proteins involved?). The final extrusion of calcium toward the plasma requires ATP driven mechanisms, whereby $PMCA_{1b}$ has a much more important role than NCX. A not fully explored mechanism might involve the calbindin mediated direct uptake into the circulation; however such mechanism would not allow the massive amount of calcium transport (>300 mg/day).

phenotype could indeed be corrected by high calcium-lactose "rescue diet" [Li et al., 1998; Amling et al., 1999]. Thus, it seemed that the primary "calcemic" target of vitamin D is to be located in the intestinal calcium absorption process rather than in bone, kidney, or parathyroid gland (Table I).

In the absence of genome wide data on gene/protein expression in the intestine of VDR KO versus Wild Type (WT) mice, a candidate gene approach can identify three potential levels of molecular action of vitamin D [Wasserman, 1997]:

1. luminal calcium influx at the brush border membrane. This process was considered to be "easy" as it shows a downhill gradient between the mmolar luminal and micromolar intracellular calcium concentration and a downhill electrochemical gradient, before ECaC1 and ECaC2 were discovered as gatekeepers for calcium entry.
2. intracellular calcium transport, whereby calbindin-D_{9K} (mammals) or calbindin-D_{28K} (birds) have been well documented as vitamin D regulated proteins which reflect—although not perfectly—the net calcium absorption process.
3. calcium extrusion from the cell via a plasma membrane calcium ATPase ($PMCA_{1b}$) (or alternatively by calbindin extrusion into the circulation [Nemere et al., 1991]).

For each of these three processes proteins/genes have either been well characterized or recently been discovered.

The recent discovery [Hoenderop et al., 1999; Peng et al., 1999] of two epithelial calcium channels belonging to a new class of calcium transporters [Hoenderop et al., 1999] and the availability of vitamin D receptor KO mice allowed a more precise analysis of the relative importance of different calcium binding or transporting proteins known to be present in the intestinal cells.

The epithelial calcium channels (ECaC1 and ECaC2 nomenclature, see Table II) belong to a large class of about 20 genes of transient receptor potential (TRP) channels of which the first member was detected in Drosophila mutants with abnormalities in phototransduction of the eye. This new gene is a member of the rapidly expanding family containing at least three subclasses each with at least six members. They are involved in very different functions such as photoreceptor function as well as mediating responses to heat or pain (TRPV1 and 2) or cold/menthol (TRPM8). The two epithelial calcium channels belong to the TRP-V (vanilloid) subfamily and are distinctive by their high

TABLE I. Lessons 2002 From VDR-KO Mice and Men

Vitamin D target tissue	Function	Genes	Redundancy
Intestine	Calcium absorption	Alkaline phosphatase	
		PMCA	
		ECaC1 and 2	
Kidney	Calcium reabsorption	Calbindin D 28K	Unknown
		Calbindin D 9K	No/Yes*
		25-OHD-24-ase	
		ECaC1 and 2	
Parathyroid	PTH synthesis & secretion	PTH	Unknown
Bone osteoblast	Matrix formation and mineralization	Alkaline phosphatase	
		Osteocalcin	
		Osteopontin	
		Matrix Gla protein	Yes???
		Collagen type 1	
		osf2/cbfa 1	
		Osteoprotegerin	
		RANKL	
Osteoclast	Bone resorption	Integrin receptor	Yes
		Carbonic anhydrase	
		RANK	
Skin	Keratinocyte differentiation	>20 genes	Yes
	Hair growth and regrowth	Hairless gene?	No
Reproductive organs	Ovarian/testicular function	Aromatase	No?
Immune system	Immune surveillance		
Monocytes and Macrophages		Proliferation	
T & B cells		Differentiation	
Dendritic cells		(IL_1-IL_2-IL_{12}...)	Yes
		(IFNγ...)	

*depending on calcium content of diet.

TABLE II. TRP Family Members[a]

TRP nomenclature	Other names	Function
TRP-C (1–7)		Photoreceptor
TRP-V		
V1		Heat & capsaicin sensitive R
V2		Heat sensitive R
V3		Thermosensitive
V4		Osmosensitive
V5	ECaC1, CaT2	Ca influx
V6	ECaC2, CaT1	Ca influx
TRP-M (1–8)		Mostly unknown
M8		Cold and menthol sensitive R

[a]R, receptor (modified from Clapham et al. [2001]).

calcium selectivity when compared to other TRP members. The general protein structure of TRP channels include a predicted six transmembrane spanning domains with a short hydrophobic stretch between S5 and S6 representing the pore region. ECaC1 is a 729 amino-acid protein present in several tissues but especially kidney, small intestine, pancreas, brain, testis, prostate and colon. All tissues with immunodetectable ECaC1 expression also express a cytosolic calcium binding protein: calbindin-D_{9K} or calbindin-D_{28K} [Müller et al., 2000]. Immunochemical localisation of ECaC1 is clearly limited to the brush border region of the intestine or apical membrane of the distal nephron.

ECaC2, originally described as CaT1 was cloned from intestinal mucosa [Peng et al., 1999] and shows 80% homology at the amino acid level with hECaC1 and this homology is greatest for the membrane spanning and pore region domains [Peng et al., 2000]. Patch clamp studies demonstrate that the characteristics of ECaC2 are comparable to those measured for ECaC1, but its expression pattern is different. Initial Northern blot analysis demonstrated that ECaC2 is predominantly expressed in small intestine, additionally this channel was detected in human placenta, pancreas, kidney, salivary gland and prostate. Interestingly ECaC1 and ECaC2 originate from two distinct genes juxtaposed with a distance of approximately 20 kb on chromosome 7q35, suggesting an evolutionary gene duplication.

Calbindin-D_{9K} and -D_{28K} have been known for many years. They are present in high cytosolic concentrations (millimolar range), which contributes to their eminent calcium binding capacity. This calcium binding activity is based on the presence of EF hands: calbindin-D_{9K} binds two calcium ions and calbindin-D_{28K} four calcium ions. Both proteins are not structurally related. Calbindin-D_{28K} is mainly expressed in the mammalian kidney and calbindin-D_{9K} primarily in the mammalian gut. However, expression of both proteins is found in mouse kidney and calbindin-D_{28K} represents the intestinal calcium binding protein in birds. Calbindin is virtually absent in vitamin D deficient animals and appears within 2–3 hr after injection of 1,25-$(OH)_2D_3$. The hypothesis is that calbindins facilitate the diffusion of calcium through the cytosol and simultaneously serve as an intracellular calcium buffer to keep the free cytoplasmic calcium concentrations below toxic levels during periods of stimulated transcellular calcium transport. A third possibility is that calbindin stimulates calcium extrusion via the plasma membrane calcium ATPase by binding to the regulatory calmodulin binding domain of this PMCA. Direct correlations between the mucosal concentration of calbindin and the efficiency of calcium absorption under a wide variety of experimental conditions have been demonstrated and support a role of the calbindins in vitamin D-mediated calcium absorption: these include adaptation to low calcium or low phosphorus diet, growth and ageing. Nevertheless, there are situations where non-correlations exist. These non-correlations can be interpreted in terms of the multifunctional role of 1,25-$(OH)_2D$ on calcium absorption and suggests that under certain circumstances a vitamin D-dependent reaction or factor other than the calbindins become limiting [Wasserman, 1997].

The extrusion of calcium from the enterocyte occurs against a steep electrochemical gradient. It has been shown experimentally that the extrusion capacity is more than adequate, and

therefore this step does not appear to be rate limiting in the overall transport [Slepchenko and Bronner, 2001]. Two active calcium transporters are present in the basolateral membrane: the PMCA (plasma membrane calcium ATPase or CaATPase protein) and the NCX (Na^+/Ca^{2+} exchanger). It was estimated that NCX in rat intestine might account for only 20% of the calcium extrusion capacity of the basolateral membrane. Vitamin D does not affect NCX activity. PMCA uses the energy stored in ATP in order to pump calcium out of the cell. It has been found in all mammalian cells and is encoded by four-independent genes. The number of isoforms is further increased by alternative splicing at two independent sites: transcripts for more than 30 isoforms have been detected. $PMCA_1$ and $PMCA_4$ are expressed quite ubiquitous, suggesting a role in cellular calcium housekeeping. However $PMCA_{1b}$ and $PMCA_{4b}$ are shown to participate in calcium reabsorption in renal distal convoluted tubule cells. Moreover, $PMCA_{1b}$ is the predominant isoform expressed in rabbit small intestine, also picked up in chicken intestine and assumed to be the isoform that participates in the calcium absorption process. Repletion of vitamin D-deficient chickens previously demonstrated a temporarily increase of (1) ATP dependent uptake of calcium by isolated basolateral membrane vesicles, (2) PMCA mRNA expression, (3) PMCA protein expression. Moreover PMCA mRNA and protein synthesis were also greater in chickens adapted to a low calcium or phosphorus diet, as compared to the normally fed control group.

Using VDR KO and WT animals we have tried to identify which of the candidate genes are most affected by 1,25-$(OH)_2D$ in order to characterise the critical steps in vitamin D-mediated calcium absorption [Van Cromphaut et al., 2001]. The two mouse strains studied were described previously [Tokyo strain: Yoshizawa et al., 1997; Leuven strain: Van Cromphaut et al., 2001] and display all the typical characteristics of vitamin D resistance: high circulating levels of 1,25-$(OH)_2D_3$, coincident with hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, failure to thrive, rickets and a progressive alopecia.

The intestinal calcium absorption was measured by a non-invasive in vivo technique. Appearance of radio-labelled calcium in serum was measured between 2 and 10 min after oral gavage. Early time points were chosen and calcium concentration in the test solution was maintained deliberately low (0.1 mM) in order to favour active over passive calcium uptake. Although this technique did not allow to quantify calcium absorption exactly, it provided useful semi-quantitative data. The calcium appearance was frankly impaired in both Leuven and Tokyo VDR KO and the 10 min plasma level was about threefold lower than in the corresponding wild type animals fed the same calcium diet (Fig. 2). In fact the ^{45}Ca area-under-the-uptake-curve (0–10 min) in Leuven VDR-KO mice,

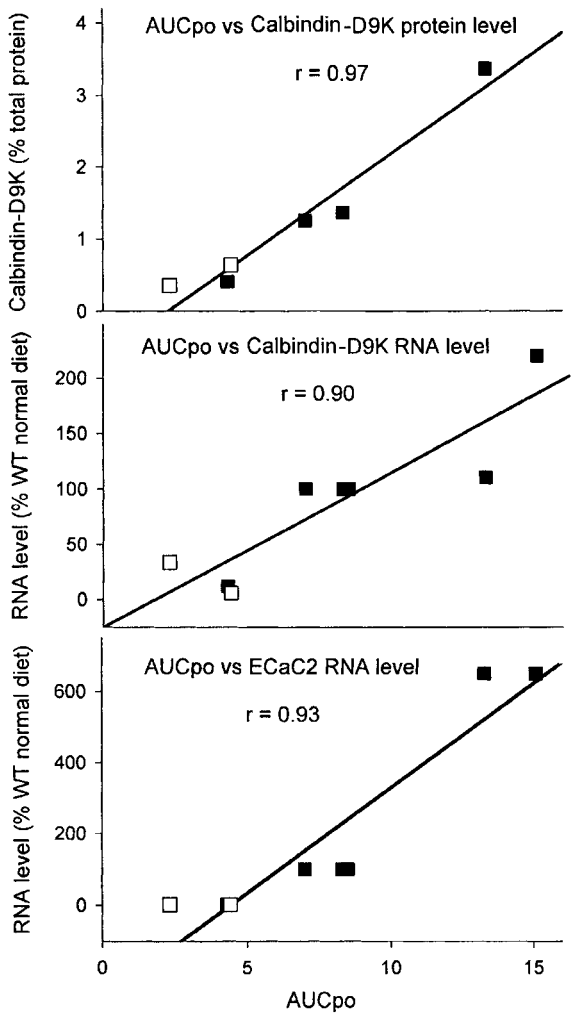

Fig. 2. Active intestinal calcium uptake and calbindin-D_{9K} and ECaC2 expression. Active intestinal calcium absorption was measured by rapid serum appearance of ^{45}Ca given orally and the area under the 0–10 min time curve (AUCpo) was calculated [Van Cromphaut et al., 2001] and compared to the mRNA (calbindin-D_{9K}, ECAC2) or protein (calbindin-D_{9K}) content of the duodenal mucosa of similar groups of animals. Open squares: VDR-KO mice (Tokyo, Leuven). Solid squares: WT mice on different calcium diets or after single injection of 1,25-$(OH)_2D$.

maximally stimulated by a low calcium diet and high 1,25-$(OH)_2$D and PTH levels was not different from the uptake in WT animals fed a high calcium diet and a 20-fold lower PTH and a 100-fold lower 1,25-$(OH)_2$D level (Fig. 2).

For the mRNA studies quantitative Real time RT-PCR technique was used. Calbindin-D_{9K} protein levels were assessed via RIA. $PMCA_{1b}$ expression levels in both KO strains were comparable to WT littermates. In duodenum of Tokyo KO mice receiving a normal diet, calbindin-D_{9K} RNA ($P < 0.01$) and protein ($P < 0.001$) levels were significantly reduced to a third, as previously described [Yoshizawa et al., 1997]. However, this deficient calbindin expression could not be confirmed in Leuven VDR KO mice although calbindin RNA levels were slightly reduced (-38%; $P < 0.001$), no significant reduction of calbindin protein level was detected. Hence the phenotype of vitamin D resistance could not be attributed to a defect in duodenal intracellular calcium binding capacity in the Leuven strain. In contrast, duodenal ECaC2 (Fig. 3A) expression was dramatically and consistently down-regulated in both Leuven ($P < 0.001$) and Tokyo ($P < 0.001$) VDR KO mice to less than 10% of WT animals. ECaC1 level was also significantly lower in duodenum of VDR KO mice ($P < 0.001$) (Fig. 3B). However, detection of ECaC2 RNA in duodenum was markedly higher (200-fold higher than ECaC1), which suggests that ECaC2 is the dominant epithelial calcium channel in duodenum as far as RNA levels are concerned in mice. Thus, surprisingly, in VDR KO mice the most affected step in transcellular calcium absorption at RNA level was calcium influx. These observations may change the whole concept of transcellular calcium absorption. Previously, on ion microscopy, calcium entry was regarded as a passive non-regulated step because calcium appeared to enter the cell at the brush border level in vitamin D-deficient chickens [Fullmer et al., 1996]. In this model, intracellular calcium binding and shuttling were considered rate-limiting. Calcium fluxes through ECaC1 or ECaC2 are blocked by a rise in intracellular calcium concentrations [Hoenderop et al., 2002]. Hence, buffering of calcium by calbindin could be indispensable for the proper functioning of ECaC. In view of this new concept, this may imply a more complex regulation driven by the interaction between calcium influx and calbindin. This concept is supported by a mathematical model of transcellular calcium transport in rat duodenum [Slepchenko and Bronner, 2001]. This model indicates the coexistence of two regulatory mechanisms of calcium influx trough the epithelial calcium channels that operate at the brush border: first, a facilitated transporter mechanism with calcium binding to the transporter (when luminal calcium is low:1–5 mM). Second, a channel calcium flux regulated by intracellular calcium: at high luminal calcium transcellular calcium transport is largely a function of the intracellular concentration of calbindin-D_{9K}.

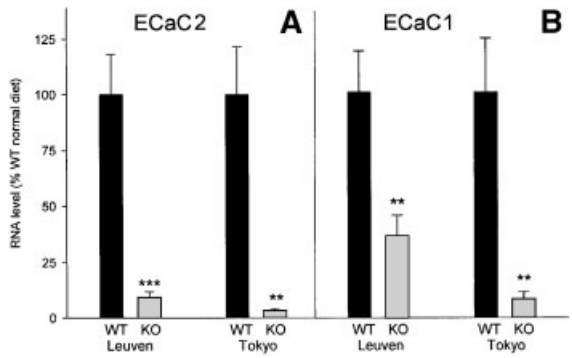

Fig. 3. Epithelial calcium channel expression in duodenum. The relative expression of ECaC2 (**panel A**) and ECaC1 (**panel B**) in VDR-KO mice and their corresponding WT mice fed a normal calcium diet, is shown. The ECaC1 duodenal expression is more than 100-fold lower than the ECaC2 mRNA concentration and therefore the relative further decrease of ECaC1 in VDR-KO mice is only an approximation.

To evaluate the effect of dietary calcium on intestinal gene expression, the candidate gene mRNA levels were measured in Leuven VDR WT and KO animals on either a low (0.02%), normal, or high (2% calcium and 20% lactose) calcium diet. Serum levels of 1,25-$(OH)_2$D increased from 57 ± 7 pg/ml in the WT control group, to 226 ± 28 pg/ml in WT mice on low calcium diet, and decreased to 19 ± 4 pg/ml on the rescue diet. In WT animals, ECaC2 expression varied considerably: a sixfold increase with calcium restriction ($P < 0.001$) and a 90% reduction with calcium abundance ($P < 0.001$), compared to levels of WT mice on normal diet (Fig. 4A). As mentioned, expression levels were quite low for ECaC1, but the tendency was comparable to findings for ECaC2: ECaC1 expression doubled ($P < 0.001$) on the low calcium diet and was reduced to 20% on the rescue diet ($P < 0.001$). RT-PCR analysis of calbindin-D_{9K} RNA levels revealed no significant difference

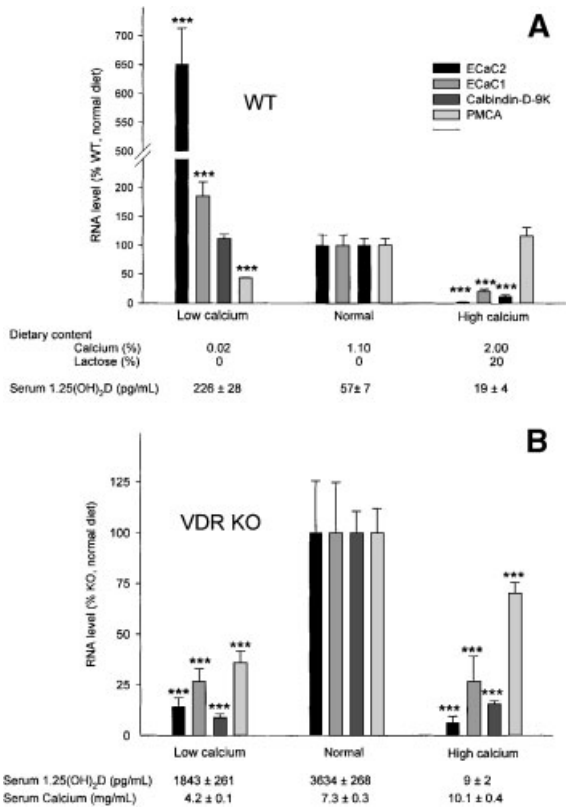

Fig. 4. Relative duodenal expression of calcium transporting proteins in WT (**panel A**) or VDR-KO (**panel B**) mice as influenced by dietary calcium intake.

between normal and low calcium diet, but a 2.5-fold increase of calbindin-D_{9K} protein content was found by RIA in WT mice on the low calcium diet ($P < 0.001$). Conversely, a more than 70% decrease in calbindin-D_{9K} RNA ($P < 0.001$) and protein level ($P < 0.05$) was found in WT animals consuming the rescue diet. $PMCA_{1b}$ gene expression was halved ($P < 0.001$) in WT on the low calcium diet.

In the KO animals on a high and on a low calcium diet, calbindin-D_{9K} RNA level decreased to less than 20% of KO on a normal diet ($P < 0.001$). This resulted in a reduction of calbindin-D_{9K} protein content of 70% on the rescue diet and of 40% on the low calcium diet, compared to levels of KO mice on a normal diet. Second, $PMCA_{1b}$ gene expression was decreased two to three times ($P < 0.001$) in KO on both low and high calcium diet. Third, in KO animals, ECaC2 and ECaC1 expression was severely impaired ($P < 0.001$) but was even further down-regulated on the low and high calcium diet to less than 25% (Fig. 4B) of the level of KO mice on normal diet.

It is generally accepted that on the one hand active intestinal calcium absorption, stimulated by 1,25-$(OH)_2$D is primordial during periods of low calcium intake. On the other hand, during periods of high calcium supply, the drive for active calcium absorption would be low as the bulk of calcium is absorbed via the paracellular pathway. When comparing the expression patterns, it is clear that for adaptation to calcium restriction an intact 1,25-$(OH)_2$D–VDR axis is indispensable: expression of the epithelial calcium channels and to a lesser extend calbindin-D_{9K} protein was solely induced in WT animals. Surprisingly, $PMCA_{1b}$ expression is down-regulated on the low calcium diet in both VDR WT and KO animals. Concerning the rescue diet, regardless of a functional VDR, the expression levels of ECaC2, ECaC1 and calbindin-D_{9K} dropped. This may imply (certainly for the VDR KO mice) a calcium-dependent VDR-independent suppressor mechanism. However, the exact nature of this mechanism is not clear.

The exact sequence of events leading to transcellular calcium transport is now better defined. The initial calcium entry may require the presence of epithelial calcium channels (especially ECaC2 or TRPV6) which belong to a new class of TRP channels. The functional properties of these channels clearly confirm their high selectivity for calcium [Hoenderop et al., 2002], but also their dependency on intracellular calcium buffering. The excellent correlation between functional measurement of calcium uptake and Calbindin-D_{9K} and ECaC2 expression (Fig. 2) suggest that both proteins are essential. Further studies in animals with selective over- or underexpression of each calcium transporter (or combined deficiency) will be needed to define the relative contribution of each protein in the chain of events. Moreover it is not unlikely that other genes/proteins are involved in the calcium uptake process. Of all presently known genes involved in intestinal calcium absorption, however, the epithelial calcium channels are the most sensitive to endogenous 1,25-$(OH)_2$D. The implication of these data for human intestinal or metabolic bone diseases are also still to be explored.

In conclusion, calcium absorption is impaired in VDR KO mice. Expression of the recently described epithelial calcium channels ECaC1 and ECaC2 is VDR-dependent and markedly down-regulated in several strains of VDR KO mice. This expression fluctuates in VDR WT

mice according to exogenous administration or dietary calcium driven variations in serum 1,25-$(OH)_2D$. Among the known active calcium absorption mechanisms, calcium influx, probably interacting with intracellular calcium transfer mechanisms, should be considered as a rate-limiting step in the process of vitamin D-dependent duodenal calcium absorption.

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