Regulation of the epithelial Ca\textsuperscript{2+} channels in small intestine as studied by quantitative mRNA detection

Monique van Abel, Joost G. J. Hoenderop, Annemiete W. C. M. van der Kemp, Johannes P. T. M. van Leeuwen, and René J. M. Bindels

Department of Cell Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands; and Department of Internal Medicine, Erasmus Medical Center Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

Submitted 21 January 2003; accepted in final form 26 February 2003

Van Abel, Monique, Joost G. J. Hoenderop, Annemiete W. C. M. van der Kemp, Johannes P. T. M. van Leeuwen, and René J. M. Bindels. Regulation of the epithelial Ca\textsuperscript{2+} channels in small intestine as studied by quantitative mRNA detection. Am J Physiol Gastrointest Liver Physiol 285: G78–G85, 2003. First published March 5, 2003; 10.1152/ajpgi.00036.2003. The epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6 are localized to the brush border membrane of intestinal cells and constitute the postulated rate-limiting entry step of active Ca\textsuperscript{2+} absorption. The aim of the present study was to investigate the hormonal regulation of these channels. To this end, the effect of 17\textbeta;-estradiol (17\textbeta;-E\textsubscript{2}), 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}), and dietary Ca\textsuperscript{2+} on the expression of the duodenal Ca\textsuperscript{2+} transport proteins was investigated in vivo and analyzed using real-time quantitative PCR. Supplementation with 17\textbeta;-E\textsubscript{2} increased duodenal gene expression of TRPV5 and TRPV6 but also calbindin-D\textsubscript{9K} and plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA1b) in ovariectomized rats. 25-Hydroxyvitamin D\textsubscript{3}-1\alpha-hydroxylase (1\alpha-OHase) knockout mice are characterized by hyperparathyroidism, rickets, hypocalcemia, and undetectable levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} and were used to study the 1,25(OH)\textsubscript{2}D\textsubscript{3}-dependency of the stimulatory effects of 17\textbeta;-E\textsubscript{2}. Treatment with 17\textbeta;-E\textsubscript{2} upregulated mRNA levels of duodenal TRPV6 in these 1\alpha-OHase knockout mice, which was accompanied by increased serum Ca\textsuperscript{2+} concentrations from 1.69 ± 0.10 to 2.03 ± 0.12 mM (P < 0.05). In addition, high dietary Ca\textsuperscript{2+} intake normalized serum Ca\textsuperscript{2+} in these mice and upregulated expression of genes encoding the duodenal Ca\textsuperscript{2+} transport proteins except for PMCA1b. Supplementation with 1,25(OH)\textsubscript{2}D\textsubscript{3} resulted in increased expression of TRPV6, calbindin-D\textsubscript{9K}, and PMCA1b and normalization of serum Ca\textsuperscript{2+}. Expression levels of duodenal TRPV5 mRNA are below detection limits in these 1\alpha-OHase knockout mice, but supplementation with 1,25(OH)\textsubscript{2}D\textsubscript{3} upregulated the expression to significant levels. In conclusion, TRPV5 and TRPV6 are positively involved in the regulation of active Ca\textsuperscript{2+} absorption in vivo. Additionally, high dietary Ca\textsuperscript{2+} intake is required for normal duodenal Ca\textsuperscript{2+} transport.

THE MAINTENANCE OF THE EXTRACELLULAR Ca\textsuperscript{2+} concentration is important for mammalian development and function. Intestinal Ca\textsuperscript{2+} absorption is a crucial control system in the regulation of Ca\textsuperscript{2+} homeostasis, because it facilitates the entry of dietary Ca\textsuperscript{2+} into the extracellular compartment.

The intestinal absorption of Ca\textsuperscript{2+} follows two pathways: a transcellular and a paracellular route. Paracellular transport is the passive, nonsaturable way of intestinal Ca\textsuperscript{2+} absorption, which occurs down an electrochemical gradient. Transcellular Ca\textsuperscript{2+} absorption takes place against an electrochemical gradient and, therefore, requires energy. This active Ca\textsuperscript{2+} transport is under the control of hormones in a Ca\textsuperscript{2+}-dependent manner. 1,25-Dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}), the active form of vitamin D, is the primary regulator of active Ca\textsuperscript{2+} absorption. 1,25(OH)\textsubscript{2}D\textsubscript{3} is synthesized from the inactive metabolite 25-hydroxvitamin D\textsubscript{3} by 25-hydroxyvitamin D\textsubscript{3}-1\alpha-hydroxylase (1\alpha-OHase) in kidney. 1,25(OH)\textsubscript{2}D\textsubscript{3} acts through nuclear vitamin D receptors (VDR), which are present within the enterocytes of the intestine. The importance of the hormones involved in Ca\textsuperscript{2+} homeostasis is reflected by severe disorders. For example, mutations in the genes encoding for 1\alpha-OHase or VDR result in pseudovitamin D-deficiency rickets (VDDR-1) and hereditary hypocalcemic vitamin D-resistant rickets (VDDR-2), respectively. In addition, high oral doses of Ca\textsuperscript{2+} can prevent the concomitant bone pathology. Furthermore, estrogen deficiency in postmenopausal women results in a negative Ca\textsuperscript{2+} balance and osteoporosis. This is often associated with intestinal malabsorption, which is corrected by estrogen therapy. On the basis of these data, it is obvious that active Ca\textsuperscript{2+} absorption in the small intestine plays an indispensable role in Ca\textsuperscript{2+} homeostasis and bone mineralization.
Active Ca\(^{2+}\) absorption is localized to the duodenum and can be described in three sequential cellular steps: entry, intracellular diffusion, and extrusion (49). The Ca\(^{2+}\)-binding protein calbindin-D\(_{9k}\) (CaBP-D\(_{9k}\)) (18, 34). The extrusion of Ca\(^{2+}\) across the basolateral membrane from the enterocyte is mediated by Ca\(^{2+}\) entry, intracellular diffusion, and extrusion (49). The molecular nature of the apical Ca\(^{2+}\) entry channel was elusive until the identification of the epithelial Ca\(^{2+}\) channels ECaC1 and ECaC2 (22, 39). These two Ca\(^{2+}\) channels represent a new family of Ca\(^{2+}\)-selective ion channels belonging to the superfamily of transient receptor potential (TRP) channels. The TRP family can be divided by sequence homology in several subfamilies (31). ECaC1 and ECaC2 are members of the TRP-Vanilloid (TRPV) subfamily and have, therefore, been renamed into TRPV5 and TRPV6, respectively (32). Both channels are expressed in several tissues including the small intestine, in which they are localized to the brush border membrane of intestinal absorptive cells (22, 53). Importantly, it has been postulated that these channels form the rate-limiting step in transcellular Ca\(^{2+}\) (re)absorption (23).

The regulation of TRPV5 and TRPV6 in duodenum may shed new light on hormone-controlled Ca\(^{2+}\) metabolism. Primary or secondary involvement of one or both epithelial Ca\(^{2+}\) channels can be expected in several pathological situations, such as VDDR and osteoporosis. Therefore, the present study was designed to investigate the regulation of TRPV5 and TRPV6 as the entry channels of active Ca\(^{2+}\) absorption in duodenum. To this end, the effects of 17β-E\(_2\), 1,25(OH)\(_2\)D\(_3\), and dietary Ca\(^{2+}\) on the expression of these duodenal Ca\(^{2+}\) transport proteins were investigated in vivo and analyzed using real-time quantitative PCR.

MATERIALS AND METHODS

Animals. Twenty-five virgin female Wistar rats (Hsd/Cpd: Wu, SPF-bred by Harlan, CPB, Zeist, The Netherlands) were subjected to a bilateral ovariectomy or sham operation. Thereafter, rats received daily 17β-E\(_2\) (Sigma, St. Louis, MO) or vehicle (gelatin, mannitol) added to the pelleted food. Sham-operated animals (Sham, n = 5) served as controls. Ovariectomized animals were given either the vehicle alone (OVX, n = 5) or 2 × 32 (OVX + E\(_2\), n = 5), 2 × 125 (OVX + E\(_2\), n = 5), or 2 × 500 μg 17β-E\(_2\)/day (OVX + E\(_2\)H, n = 5). Treatment was started immediately after ovarioectomy and lasted for 7 days.

1α-OHase knockout mice were generated by Dardenne and colleagues (14) through inactivation of the 1α-OHase gene. Three different experiments were performed, using these homozygous knockout mice as a vitamin D-deficient model, to study the effect of: 1) 17β-E\(_2\) supplementation: using Alzet osmotic minipumps (model 1007D). Eight male 1α-OHase knockout mice, 9 wk of age, were randomized in two groups. Control mice received vehicle solution alone [15% (vol/vol) ethanol, 50% (vol/vol) DMSO], and the supplemented group received an infusion dose of 10 μg 17β-E\(_2\)/day for 7 days; 2) Ca\(^{2+}\) supplementation: eight 1α-OHase knockout mice were equally divided into two groups and were fed either a normal diet [1.1% (wt/wt) Ca\(^{2+}\)] or DMSO, and the supplemented group received a Ca\(^{2+}\}-enriched diet (4% (wt/wt) Ca\(^{2+}\), 0.8% (wt/wt) phosphorus, 0% (wt/wt) lactose) from ages 3 to 8 wk or received a Ca\(^{2+}\)-enriched diet (2% (wt/wt) Ca\(^{2+}\), 1.25% (wt/wt) phosphorus, 20% (wt/wt) lactose; Harlan Tekland, Madison, WI); 3) 1,25(OH)\(_2\)D\(_3\) supplementation: eight 1α-OHase knockout mice receiving either 1,25(OH)\(_2\)D\(_3\) or vehicle injections intraperitoneally from ages 3 to 8 wk. From weeks 3 to 4, mice were daily injected intraperitoneally with 1,25(OH)\(_2\)D\(_3\) repletions of 500 and 100 μg/kg body wt daily in weeks 5–8.

At the end of the treatment periods, animals were killed and blood and duodenum tissue samples were taken. The animal ethics board of the University Medical Center Nijmegen approved all animal experimental procedures.

Analytical procedures. Serum Ca\(^{2+}\) concentrations were analyzed using a colorimetric assay kit as described previously (5). Serum 17β-E\(_2\) was measured by an extraction procedure using diethyl ether followed by radioimmunoassay (DPC, Los Angeles, CA) (13).

RNA isolation and quantitative PCR. Total RNA from duodenal mucosa was isolated using TRIzol reagent (GIBCO-BRL, Life Technologies, Breda, The Netherlands) according to the manufacturer’s protocol. RNA was treated with DNase to prevent contamination of genomic DNA and finally resuspended in diethylpyrocarbonate-treated miliQ. Total RNA (2 μg) was subjected to reverse transcription using Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) as described previously (22). Expression levels of duodenal TRPV5, TRPV6, calbindin-D\(_{9k}\), and PMCA1b mRNA were quantified by real-time quantitative PCR, using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA).

Table 1. Sequences of primers and Taqman probes for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV5</td>
<td>5′-CTTAGCGGCTTGAGACCAAGA-3′</td>
<td>5′-GGGCGTCACTGCTTATCA-3′</td>
<td>5′-GCTCTTGAATAAGGTTTGGTCA-3′</td>
</tr>
<tr>
<td>TRPV6</td>
<td>5′-CGTTCTAGGAGCAAAACATAC-3′</td>
<td>5′-ATGGTTTCTCGTATAGTACCT-3′</td>
<td>5′-GCTCAGAGAGGATTCAGAC-3′</td>
</tr>
<tr>
<td>CaBP-D(_{9k})</td>
<td>5′-GCTACGGAGGACCAAGAAGCC-3′</td>
<td>5′-GGTGCCAGCTGAGATAGGTC-3′</td>
<td>5′-GGTGCCAGCTGAGATAGGTC-3′</td>
</tr>
<tr>
<td>PMCA1b</td>
<td>5′-GGTCCTGATTCTGAAGAAC-3′</td>
<td>5′-GGTCTCTGATTCTGAAGAAC-3′</td>
<td>5′-GGTCTCTGATTCTGAAGAAC-3′</td>
</tr>
<tr>
<td>HPR7</td>
<td>5′-ATGCGACCACAGGCAACAGGAC-3′</td>
<td>5′-GGTCTCTGATTCTGAAGAAC-3′</td>
<td>5′-GGTCTCTGATTCTGAAGAAC-3′</td>
</tr>
</tbody>
</table>

PCR primers and fluorescent probes (5′ FAM-3′ TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Bioulie (Malden, The Netherlands). Transect receptor potential vanilled, TRPV5/6, epithelial Ca\(^{2+}\) channel 1 and 2; CaBP-D\(_{9k}\), calbindin-D\(_{9k}\); PMCA1b, plasma membrane Ca\(^{2+}\)-ATPase; HPR7: hypoxanthine-guanine phosphoribosyl transferase. R, rat; M, mouse.
was calculated and expressed as a ratio to the hypoxanthine-guanine phosphoribosyl transferase gene. Primers and probes targeting the genes of interest were designed using Primer Express software (Applied Biosystems, Foster City, CA) and are listed in Table 1.

Statistical analysis. Values are expressed as means ± SE. Statistical significance was determined by ANOVA followed by contrast analysis according to Fisher. In the case of only two experimental groups, statistical significance was determined using the Mann-Whitney U-test. Differences in means with \( P \) values <0.05 were considered statistically significant. All analyses were performed using the Statview Statistical Package (Power PC version 4.5.1, Berkeley, CA) on a Macintosh computer.

RESULTS

OVX Wistar rats were used as a model of estrogen deficiency. Ovariectomy was confirmed by the reduced serum 17β-E2 levels compared with Sham-operated animals (Table 2). Correction of this deficiency by supplementation with 17β-E2 resulted in a dose-responsive increase with significantly higher serum 17β-E2 levels in OVX + E2H rats (Table 2). Importantly, 17β-E2 treatment reduced serum \( \text{Ca}^{2+} \) levels, resulting in a slight but significantly lower serum \( \text{Ca}^{2+} \) concentration in the OVX + E2H group (Table 2).

Subsequently, we investigated whether 17β-E2 treatment altered the expression of genes encoding \( \text{Ca}^{2+} \) transport proteins involved in duodenal transcellular \( \text{Ca}^{2+} \) absorption. With the use of real-time quantitative PCR, a more than sevenfold increase in TRPV6 mRNA levels was observed in OVX rats supplemented with the highest dose of 17β-E2 compared with untreated OVX animals (Fig. 1A). TRPV5 gene expression was also upregulated by 17β-E2, although detection levels were lower and differences between the various groups were less pronounced than for TRPV6 (Fig. 1B). In addition, upregulation of both \( \text{Ca}^{2+} \) channels was accompanied by an increase in expression of the other \( \text{Ca}^{2+} \) transport proteins, namely calbindin-D9K (9-fold) and PMCA1b (2-fold, Fig. 1, C and D).

These observations led us to study the influence of 17β-E2 treatment on duodenal TRPV5 and TRPV6 expression in \( 1\alpha \)-OHase knockout mice to investigate the involvement of 1,25(OH)\(_2\)D\(_3\). Serum 17β-E2 levels were not detectable in the male mice but rose to 67 pg/ml after treatment with 17β-E2. Interestingly, after

---

**Table 2. Effects of OVX and 17β-E2 supplementation on serum parameters in female Wistar rats**

<table>
<thead>
<tr>
<th>17β-E2, pg/ml</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX E(_2)L</th>
<th>OVX E(_2)M</th>
<th>OVX E(_2)H</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ca}^{2+}, \text{mM} )</td>
<td>2.38 ± 0.02</td>
<td>2.40 ± 0.03</td>
<td>2.39 ± 0.02</td>
<td>2.36 ± 0.04</td>
<td>2.31 ± 0.02</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (\( n = 5 \)). OVX, ovariectomized; 17β-E2, 17β-estradiol; Sham, sham-operated; E\(_2\)L, supplemented with 2 \( \times \) 32 µg 17β-E2/day; E\(_2\)M, supplemented with 2 \( \times \) 125 µg 17β-E2/day; E\(_2\)H, supplemented with 2 \( \times \) 500 µg 17β-E2/day. *\( P < 0.05 \) vs. OVX. †\( P < 0.05 \) vs. all groups.
treatment with 17β-E2, serum Ca²⁺ levels significantly increased from a hypocalcemic state to subnormal concentrations of 2.03 ± 0.12 mM (Table 3). Analysis of gene expression in duodenum revealed a 12-fold increase in TRPV6 mRNA after treatment with 17β-E2 (Fig. 2).

In two following experiments, the 1α-OHase knockout mice were used to study the influence of 1,25(OH)₂D₃ itself and dietary Ca²⁺ on gene expression levels of the Ca²⁺ transport proteins. Inactivation of the 1α-OHase gene in the knockout mice resulted in severe hypocalcemia with serum Ca²⁺ concentrations as low as 1.20 mM. Supplementation with 1,25(OH)₂D₃ or a high dietary Ca²⁺ intake normalized serum Ca²⁺ concentrations (Table 3). Subsequently, analysis of gene expression showed an increase in mRNA levels of TRPV6 after high dietary Ca²⁺ intake (Fig. 3A). 1,25(OH)₂D₃ supplementation also upregulated the expression of this transcript, but to a much higher degree (Fig. 4A). In addition, high dietary Ca²⁺ stimulated the expression of calbindin-D₉K significantly (Fig. 3B), whereas PMCA1b levels were not significantly changed (P > 0.1; Fig. 3C). Supplementation with 1,25(OH)₂D₃ significantly upregulated the expression of both calbindin-D₉K (Fig. 4B) and PMCA1b (Fig. 4C).

Table 3. The effect of 17β-E₂, 1,25(OH)₂D₃, and high dietary Ca²⁺ on serum Ca²⁺ levels in 1α-OHase knockout mice

<table>
<thead>
<tr>
<th>Serum Ca²⁺ Concentration, mM</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-E₂</td>
<td>1.69 ± 0.10</td>
<td>2.03 ± 0.12*</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>1.20 ± 0.05</td>
<td>2.59 ± 0.07*</td>
</tr>
<tr>
<td>High dietary Ca²⁺</td>
<td>1.20 ± 0.05</td>
<td>2.33 ± 0.10*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 4). 1α-OHase, 25-hydroxyvitamin D₃-1α-hydroxylase; control, 1α-OHase knockout mice; treated, 1α-OHase knockout mice supplemented with 17β-E₂ (10 μg/day for 7 days), 1,25(OH)₂D₃ (500 pg/g body weight daily in weeks 3–4 and 100 pg/g body weight daily in weeks 5–8), or high dietary Ca²⁺ (2% wt/wt) Ca²⁺-enriched diet from age 3–8 wk). *P < 0.05 vs. control.

Fig. 2. Effect of 17β-E₂ supplementation on the mRNA expression level of TRPV6 in duodenum of 25-hydroxyvitamin D₃-1α-hydroxylase (1α-OHase) knockout mice. Duodenal expression of TRPV6 assessed by real-time quantitative PCR analysis is presented as a ratio to HPRT expression. Control, 1α-OHase knockout mice; 17β-E₂, 1α-OHase knockout mice supplemented with 10 μg 17β-E₂/day. Data are presented as means ± SE (n = 4). *P < 0.05 vs. control.

Fig. 3. Effect of dietary Ca²⁺ on the mRNA expression levels of Ca²⁺ transport proteins in duodenum of 1α-OHase knockout mice. Duodenal expression of TRPV6 (A), calbindin-D₉K (B), and PMCA1b (C) of the different experimental groups assessed by real-time quantitative PCR analysis is presented as a ratio to HPRT expression. Control, 1α-OHase knockout mice; calcium, 1α-OHase knockout mice on a Ca²⁺-enriched diet [2% (wt/wt) Ca²⁺ from ages 3–8 wk]. Data are presented as means ± SE (n = 4). *P < 0.05 vs. control.
OVX rats were used as an animal model of estrogen deficiency in postmenopausal women (29). Ovariectomy did not affect mRNA expression levels of the various Ca\(^{2+}\) transport proteins in duodenum, which agrees with the measured unchanged serum Ca\(^{2+}\) levels 1 wk after OVX. Theoretically, the loss of function of 17\(\beta\)-E\(_2\) could be compensated by other mechanisms such as 1,25(OH)\(_2\)D\(_3\) within a 7-day period. In contrast, a significant upregulation of TRPV5 and TRPV6 mRNA expression was observed after estrogen replacement therapy in OVX rats. These increased mRNA levels were accompanied by upregulated mRNA levels of both calbindin-D\(_{9k}\) and PMCA1b. However, upregulation of the genes encoding for the Ca\(^{2+}\) transport proteins was accompanied by decreased serum Ca\(^{2+}\) levels after 17\(\beta\)-E\(_2\) treatment. Several studies in human subjects observed this effect of estrogen treatment on serum Ca\(^{2+}\) levels (16, 41). It has been suggested that this fall in Ca\(^{2+}\) is transitory, due to increased Ca\(^{2+}\) requirements of the estrogen-deficient animals. Correction of estrogen deficiency results in decreased bone resorption and increased formation, causing a slight fall in serum Ca\(^{2+}\) concentration (40).

Because 1,25(OH)\(_2\)D\(_3\) is the primary hormone involved in the regulation of Ca\(^{2+}\) absorption, it has been suggested that the effects of estrogen on intestinal absorption of Ca\(^{2+}\) are indirectly mediated by 1,25(OH)\(_2\)D\(_3\) (40). In the kidney, production of 1,25(OH)\(_2\)D\(_3\) by 1a-OHase plays a pivotal role in maintaining Ca\(^{2+}\) homeostasis (52). It was demonstrated by Stumpf et al. (44) that 17\(\beta\)-E\(_2\) was retained in the cell nuclei of proximal tubules, where the synthesis of 1,25(OH)\(_2\)D\(_3\) takes place. Conflicting data are presented concerning the effect of 17\(\beta\)-E\(_2\) on 1a-hydroxylase activity and 1,25(OH)\(_2\)D\(_3\) synthesis (1, 11–13, 16, 20). So far, conclusive in vivo data for a direct effect of 17\(\beta\)-E\(_2\), independent of 1,25(OH)\(_2\)D\(_3\), on intestinal Ca\(^{2+}\) absorption are lacking.

Dardenne et al. (14) generated 1\(\alpha\)-OHase knockout mice by targeted inactivation of the 1\(\alpha\)-OHase gene. These knockout mice express the same clinical phenotype as patients with VDDR-1, characterized by hyperparathyroidism, hypocalcemia, rickets, and undetectable levels of 1,25(OH)\(_2\)D\(_3\). These mice represent an ideal animal model in which to study the role of 17\(\beta\)-E\(_2\) on intestinal Ca\(^{2+}\) transport independent of 1,25(OH)\(_2\)D\(_3\). Treatment with 17\(\beta\)-E\(_2\) increased serum Ca\(^{2+}\) levels to subnormal concentrations. Furthermore, 17\(\beta\)-E\(_2\) treatment was associated with an upregulation of duodenal TRPV6 mRNA expression. The observations that functional estrogen receptors are present within the enterocytes (47) and that 17\(\beta\)-E\(_2\) enhances the uptake of Ca\(^{2+}\) by intestinal cells in vitro (2) are suggestive of a direct role in Ca\(^{2+}\) absorption. Together, these findings provide further evidence that 17\(\beta\)-E\(_2\) acts directly on duodenum to promote active Ca\(^{2+}\) absorption.

In the 1\(\alpha\)-OHase knockout mice, high dietary Ca\(^{2+}\) intake increased the expression levels of the genes encoding Ca\(^{2+}\) transport proteins, which was accompanied by normalization of serum Ca\(^{2+}\) levels. Under
physiological conditions, Ca\(^{2+}\) acts via a negative feedback mechanism that eventually leads to suppression of 1α-OHase activity and production of 1,25(OH\(_2\))D\(_3\), which decreases expression of the Ca\(^{2+}\)-transporting proteins and active Ca\(^{2+}\) absorption (6). However, this study suggests that in the absence of 1α-OHase activity, and thus circulating 1,25(OH\(_2\))D\(_3\), Ca\(^{2+}\) supplementation can increase the expression level of duodenal Ca\(^{2+}\) transport proteins. The mechanism that underlies this vitamin D-independent Ca\(^{2+}\)-regulated pathway is not known. Previous studies have shown that cAMP- and serum-response elements can function as a Ca\(^{2+}\)-response element (CARE) in the control of gene expression (19, 43). Recently, a new Ca\(^{2+}\)-responsive transcription factor was discovered in neuronal cells that contributes to Ca\(^{2+}\)-stimulated gene expression of the brain-derived neurotrophic factor (BDNF) through a CARE found in the promoter of the BDNF gene (45). Moreover, in the promoter region of calbindin-D\(_{28K}\), a Ca\(^{2+}\)-sensitive transcriptional regulatory mechanism, named Purkinje cell element, was identified, which may play a key role in setting the Ca\(^{2+}\)-buffering capacity of Purkinje cells (3). Likewise, Ca\(^{2+}\)-response elements and/or transcription factors could be involved in the Ca\(^{2+}\)-mediated regulation of gene expression found in our study.

Interestingly, high dietary Ca\(^{2+}\) intake, using VDR knockout mice, resulted in a decreased expression of both TRPV5 and TRPV6 and a reduction in calbindin-D\(_{28K}\) and PMCA1b expression (48). The VDR is a nuclear receptor and acts as a ligand-activated transcription factor. On activation by 1,25(OH\(_2\))D\(_3\), the VDR can alter the rate of gene expression. However, 1,25(OH\(_2\))D\(_3\) can also activate second-messenger pathways mediated by cell surface receptors (33, 35). Furthermore, previous studies (33, 36) have shown that this nongenomic effect of 1,25(OH\(_2\))D\(_3\) can stimulate intestinal Ca\(^{2+}\) transport, a process called transcaltrichia. VDR knockout mice have elevated 1,25(OH\(_2\))D\(_3\) levels, which decrease after high dietary Ca\(^{2+}\) intake (48). It is conceivable that this influences the intestinal expression of Ca\(^{2+}\)-transport proteins and Ca\(^{2+}\) absorption through 1,25(OH\(_2\))D\(_3\)-mediated nongenomic pathways, which could possibly explain the differential findings between VDR and 1α-OHase knockout mice after high dietary Ca\(^{2+}\) intake.

The genomic response of 1,25(OH\(_2\))D\(_3\) is demonstrated by the significant increase in mRNA levels of TRPV6, calbindin-D\(_{28K}\), and PMCA1b after supplementation with 1,25(OH\(_2\))D\(_3\) in the 1α-OHase knockout mice. Furthermore, repletion with 1,25(OH\(_2\))D\(_3\) normalized serum Ca\(^{2+}\) concentrations. In agreement with our findings, previous studies (9, 46, 50) have shown that 1,25(OH\(_2\))D\(_3\) stimulates the expression level of calbindins and affects Ca\(^{2+}\) extrusion at the basolateral membrane of duodenal cells. 1,25(OH\(_2\))D\(_3\) has also been shown to stimulate transcellular Ca\(^{2+}\) transport in the human intestinal cell line Caco-2 and to increase the expression of calbindin-D\(_{28K}\) in these cells (15, 17). Recently, Wood et al. (51) demonstrated that expression of TRPV6 in Caco-2 cells is upregulated by 1,25(OH\(_2\))D\(_3\). Besides TRPV6, also TRPV5 is expressed as apical Ca\(^{2+}\) channel in duodenum (22, 53). However, mRNA expression levels of this latter Ca\(^{2+}\) channel are hundredfolds lower in duodenum. Several other studies (4, 38, 51) reported that TRPV5 expression could not be detected in Caco-2 cells and human intestinal tissue. In our 1α-OHase knockout mice, the expression of duodenal TRPV5 is also below detection limits. Only after supplementation with 1,25(OH\(_2\))D\(_3\), TRPV5 mRNA reaches a detectable level in duodena of these knockout mice. Together, these findings indicate that 1,25(OH\(_2\))D\(_3\) is a significant regulator of both epithelial Ca\(^{2+}\) channels in duodenum and support the idea that 1,25(OH\(_2\))D\(_3\) stimulates active intestinal Ca\(^{2+}\) absorption by increasing the rate of Ca\(^{2+}\) influx across the intestinal brush border membrane (42, 49). In addition to TRPV6, which is abundantly present in duodenum, TRPV5 can also be strongly upregulated and could play an important role in intestinal Ca\(^{2+}\) absorption. The generation of TRPV5 and TRPV6 knockout mice will further substantiate the importance of these channels in Ca\(^{2+}\) homeostasis in general and, in particular, their role in Ca\(^{2+}\) absorption.

Similar to TRPV5 and TRPV6, calbindin-D\(_{28K}\) and PMCA1b mRNA levels are also upregulated after different supplementations in rat and mouse. The activity of the epithelial Ca\(^{2+}\)-channels is controlled by a Ca\(^{2+}\)-dependent feedback mechanism (25, 37). Therefore, to facilitate Ca\(^{2+}\) transport, it is important to maintain a low intracellular Ca\(^{2+}\) environment. By the upregulation of the buffering and extrusion mechanisms, this requirement is fulfilled. Moreover, upregulation of expression levels of the genes encoding the intestinal Ca\(^{2+}\) transport proteins was accompanied by normalization of the serum Ca\(^{2+}\) concentration. Together, these findings underline the intimate relationship among apical influx, cytosolic diffusion, and basolateral efflux systems in transcellular Ca\(^{2+}\) transport, which could contribute to increased Ca\(^{2+}\) absorption and ultimately normalization of serum Ca\(^{2+}\) levels.

In conclusion, the present study demonstrated that 17β-E\(_2\) and 1,25(OH\(_2\))D\(_3\) are both positively involved in the regulation of duodenal TRPV5 and TRPV6, whereas dietary Ca\(^{2+}\) has a stimulatory effect on the expression of TRPV6 only. This regulation substantiates the possible role of these channels in the pathogenesis of hormone-regulated Ca\(^{2+}\)-disorders, such as osteoporosis or VDDR. Future research should aim to further unravel the mechanisms controlling the activity of TRPV5 and TRPV6, which may lead to new insights regarding Ca\(^{2+}\) homeostasis-related disorders.

We thank Organon Nederland for donating duodenal tissue samples from the ovariectomized rat study and Drs. R. St-Arnaud and O. Dardennes for providing the 1α-OHase knockout mice.

This work was supported by grants from the Dutch Organization of Scientific Research (Zon-Mw 902.18.298, Zon-Mw 016.006.001).
REFERENCES


