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

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1Department of Cell Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands; and 2Department of Internal Medicine, Erasmus Medical Center Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

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Van Abel, Monique, Joost G. J. Hoenderop, Anнемиеke 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 17β-estradiol (17β-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 17β-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 ovariec-tomized rats. 25-Hydroxyvitamin D\textsubscript{3}–1α-hydroxylase (1α-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 17β-E\textsubscript{2}. Treatment with 17β-E\textsubscript{2} upregulated mRNA levels of duodenal TRPV6 in these 1α-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 1α-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 regulated by 17β-E\textsubscript{2} and 1,25(OH)\textsubscript{2}D\textsubscript{3}, whereas dietary Ca\textsuperscript{2+} is positively involved in the regulation of TRPV6 only.

ECA\textsubscript{C}; CaT\textsubscript{1}; estrogen; vitamin D; dietary Ca\textsuperscript{2+}

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 (28).

The intestinal absorption of Ca\textsuperscript{2+} follows two pathways: a transcellular and a paracellular route (49). 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 (7). 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-hydroxyvitamin D\textsubscript{3} by 25-hydroxyvitamin D\textsubscript{3}–1α-hydroxylase (1α-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 (8, 27, 46). In addition, functional estrogen receptors have also been detected in small intestine (47). Arjmandi et al. (2) showed that 17β-estradiol (17β-E\textsubscript{2}) enhances the uptake of Ca\textsuperscript{2+} by intestinal cells in vitro. Furthermore, active intestinal Ca\textsuperscript{2+} absorption can be regulated by dietary Ca\textsuperscript{2+} intake. Active absorption of Ca\textsuperscript{2+} is increased after feeding a low-Ca\textsuperscript{2+} diet or under conditions of increased Ca\textsuperscript{2+} needs (7).

The importance of the hormones involved in Ca\textsuperscript{2+} homeostasis is reflected by severe disorders. For example, mutations in the genes encoding for 1α-OHase or VDR result in pseudovitamin D-deficiency rickets (VDDR-1) and hereditary hypocalcemic vitamin D-resistant rickets (VDDR-2), respectively (26, 30). High oral doses of Ca\textsuperscript{2+} can prevent the concomitant bone pathology (21). 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 (16). 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\(9K\) is involved in intracellular diffusion of Ca\(^{2+}\). It binds Ca\(^{2+}\) and moves it from the brush border membrane to the basolateral site of the duodenal cell. In this respect, calbindin serves as both a Ca\(^{2+}\) carrier and a cytosolic Ca\(^{2+}\) buffer (18, 34). The extrusion of Ca\(^{2+}\) across the basolateral membrane from the enterocyte is mediated by the plasma membrane Ca\(^{2+}\)-ATPase PMCA1b (10). The molecular nature of the apical Ca\(^{2+}\) entry channel was elusive until the identification of the epithelial Ca\(^{2+}\) channels ECaC1 and ECaC2 (24, 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, 59). 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 17\(\beta\)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 analyzied 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 17\(\beta\)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 \(\times\) 32 (OVX + E\(_2\L\), \(n = 5\)), 2 \(\times\) 125 (OVX + E\(_2M\), \(n = 5\)), or 2 \(\times\) 500 \(\mu\)g 17\(\beta\)E\(_2/\)day (OVX + E\(_2H\), \(n = 5\)). Treatment was started immediately after ovariectomy and lasted for 7 days.

1\(\alpha\)-OHase knockout mice were generated by Dardenne and colleagues (14) through inactivation of the 1\(\alpha\)-OHase gene. Three different experiments were performed, using these homozygous knockout mice as a vitamin D-deficient model, to study the effect of: 1) 17\(\beta\)E\(_2\) supplementation: using Alzet osmotic minipumps (model 1007D). Eight male 1\(\alpha\)-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 \(\mu\)g 17\(\beta\)E\(_2/\)day for 7 days; 2) Ca\(^{2+}\) supplementation: eight 1\(\alpha\)-OHase knockout mice were equally divided into two groups and were fed either a normal diet (1.1% 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 Teklad, Madison, WI); 3) 1,25(OH)\(_2\)D\(_3\) supplementation: eight 1\(\alpha\)-OHase knockout mice received 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 \(\mu\)g 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 17\(\beta\)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 milliQ. Total RNA (2 \(\mu\)g) 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, Rotkreuz, Switzerland). With the use of standard curves, the amount of copy numbers of the target genes in each sample

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>TRPV5</td>
<td>R 5' - 5'-TTGCAGAACGCAGCAACCTCA-3'</td>
<td>5'-TTGCAGAACGCAGCAACCTCA-3'</td>
<td>5'-FAM-3'-TTCCTTGAGATGTTCTGTGATTCTCTCTTUT-3'</td>
</tr>
<tr>
<td>TRPV6</td>
<td>R 5'-ATCCCCTGGATCTCCA-3'</td>
<td>5'-ATCCCCTGGATCTCCA-3'</td>
<td>5'-FAM-3'-TTCCTTGAGATGTTCTGTGATTCTCTCTTUT-3'</td>
</tr>
<tr>
<td>CaBP-D(_{9K})</td>
<td>R 5'-GGCTGAAGAAAGAAGAGCTCTT-3'</td>
<td>5'-GGCTGAAGAAAGAAGAGCTCTT-3'</td>
<td>5'-FAM-3'-TTCCTTGAGATGTTCTGTGATTCTCTCTTUT-3'</td>
</tr>
<tr>
<td>PMCA1b</td>
<td>R 5'-CCACATGTTCTCTGACAGCT-3'</td>
<td>5'-CCACATGTTCTCTGACAGCT-3'</td>
<td>5'-FAM-3'-TTCCTTGAGATGTTCTGTGATTCTCTCTTUT-3'</td>
</tr>
<tr>
<td>HPR</td>
<td>R 5'-TTTGATGGTCTCTGACAGCT-3'</td>
<td>5'-TTTGATGGTCTCTGACAGCT-3'</td>
<td>5'-FAM-3'-TTCCTTGAGATGTTCTGTGATTCTCTCTTUT-3'</td>
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</table>

PCR primers and fluorescent probes (5 FAM-3 TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Biologie (Malden, The Netherlands). Transient receptor potential vanillic acid (TRPV5/6), epithelial Ca\(^{2+}\) channel 1 and 2 (CaBP-D\(_{9K}\), calbindin-D\(_{9K}\); PMCA1b, plasma membrane Ca\(^{2+}\)-ATPase; HPR: 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 4.51, 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 Ca2+ levels, resulting in a slight but significantly lower serum Ca2+ concentration in the OVX + E2H group (Table 2).

Subsequently, we investigated whether 17β-E2 treatment altered the expression of genes encoding Ca2+ transport proteins involved in duodenal transcellular Ca2+ 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 Ca2+ channels was accompanied by an increase in expression of the other Ca2+ 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 1α-OHase knockout mice to investigate the involvement of 1,25(OH)2D3. Serum 17β-E2 levels were not detectable in the male mice but rose to 67 pg/ml after treatment with 17β-E2. Interestingly, after

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

<table>
<thead>
<tr>
<th>Serum Parameter</th>
<th>Sham (n=5)</th>
<th>OVX (n=5)</th>
<th>OVX E2L (n=5)</th>
<th>OVX E2M (n=5)</th>
<th>OVX E2H (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-E2 (pg/ml)</td>
<td>11 ± 6</td>
<td>5 ± 3</td>
<td>5 ± 1</td>
<td>12 ± 2</td>
<td>64 ± 6†</td>
</tr>
<tr>
<td>Ca2+, 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; E2L, supplemented with 2 × 32 µg 17β-E2/day; E2M, supplemented with 2 × 125 µg 17β-E2/day; E2H, supplemented with 2 × 500 µg 17β-E2/day. †P < 0.05 vs. OVX.
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). Detection of TRPV5 mRNA in duodenum was below detection limits in the 1α-OHase knockout mice. In addition, after treatment with either 17β-E₂ or high dietary Ca²⁺, expression of TRPV5 mRNA levels could also not be detected. Interestingly, supplementation with 1,25(OH)₂D₃ upregulated the expression of TRPV5 mRNA to significant levels.

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></th>
<th>Serum Ca²⁺ Concentration, mM</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>1.69 ± 0.10</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>High dietary Ca²⁺</td>
<td>1.20 ± 0.05</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.
OVX rats were used as an animal model of estrogen deficiency in postmenopausal women (29). Ovarietomy 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 17β-E\(_2\) could be compensated by other mechanisms such 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 17β-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 1α-OHase plays a pivotal role in maintaining Ca\(^{2+}\) homeostasis (52). It was demonstrated by Stumpf et al. (44) that 17β-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 17β-E\(_2\) on 1α-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 17β-E\(_2\), independent of 1,25(OH)\(_2\)D\(_3\), on intestinal Ca\(^{2+}\) absorption are lacking.

Dardenne et al. (14) generated 1α-OHase knockout mice by targeted inactivation of the 1α-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 17β-E\(_2\) on intestinal Ca\(^{2+}\) transport independent of 1,25(OH)\(_2\)D\(_3\). Treatment with 17β-E\(_2\) increased serum Ca\(^{2+}\) levels to subnormal concentrations. Furthermore, 17β-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 17β-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 17β-E\(_2\) acts directly on duodenum to promote active Ca\(^{2+}\) absorption.

In the 1α-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

**DISCUSSION**

The present study demonstrated that duodenal TRPV5 and TRPV6 mRNA levels are both upregulated by 17β-E\(_2\) and 1,25(OH)\(_2\)D\(_3\), whereas dietary Ca\(^{2+}\) is positively involved in the regulation of TRPV6 mRNA only. Moreover, the expression of genes encoding the other known duodenal Ca\(^{2+}\) transport proteins is upregulated concomitantly, which will facilitate Ca\(^{2+}\) absorption optimally.

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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 transcalta

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.

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