Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines

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1Department of Foods and Nutrition, Purdue University, West Lafayette, Indiana 47906; 2Graduate Program in Nutrition, University of North Carolina at Greensboro, Greensboro, North Carolina 27403; and 3Mineral Bioavailability Laboratory, United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts 02111

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Fleet, James C., Faria Eksir, Kenneth W. Hance, and Richard J. Wood. Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. Am J Physiol Gastrointest Liver Physiol 283: G618–G625, 2002.—The parental cell line (P) of Caco-2 cells and two clones, BBe and TC7, were studied at 11 days postconfluence to test the facilitated diffusion model of vitamin D-mediated intestinal calcium absorption (CaTx). Nuclear vitamin D receptor (nVDR) and calbindin D9k (CaBP) were measured by Western blot; 1,25-hydroxyvitamin D3 24-hydroxylase (CYP24), plasma membrane Ca-ATPase (PMCA), and Ca transport channel (CaT1) mRNA levels were examined by RT-PCR; and net apical-to-basolateral CaTx was examined after treating cells with vehicle or 10 nM calcitriol for 8 (mRNA levels) or 48 h (protein, CaBP mRNA, CaTx). nVDR level was lowest in BBe (38% P value) and directly related to CYP24 induction (TC7 = P, which were 1.56 times greater than BBe). nVDR was inversely related to the vitamin D-induced levels of CaT1 mRNA, CaBP mRNA, PMCA mRNA, and net CaTx, with the highest induction seen in BBe. Basal CaBP mRNA (86 times greater than P) and protein levels were highest in TC7 cells and were not associated with higher net CaTx, suggesting CaBP may not be rate limiting for CaTx in these cells.

intestine; calcium absorption; calbindin D9k; calcium transport channel; plasma membrane Ca-adenosinetriphosphatase

INTESTINAL CALCIUM ABSORPTION and its regulation by vitamin D status have been studied for over 60 years (26). The active metabolite that controls this process is 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] (27). To date, two models have been proposed to explain this process: the facilitated diffusion model (2) and the vesicular model (23). In the facilitated diffusion model, summarized in Fig. 1, the basal rate of calcium uptake and calcium extrusion from the absorptive epithelial cell is proposed to be sufficient to accommodate the elevated rate of transport seen after vitamin D stimulation. In contrast, mathematical modeling shows that intracellular diffusion is the rate-limiting, as well as the most vitamin D responsive, step. Calbindin D9k has been proposed as the protein that binds calcium and facilitates its diffusion between the apical and basolateral surfaces of the cell (8). The level of calbindin D9k in the intestine is closely correlated with the efficiency of calcium absorption (2), and thus this protein plays a central role in the facilitated diffusion model. In the vesicular model, the cell uses lysosomes to sequester calcium and facilitate its movement to the basolateral membrane for extrusion (24). While calbindin D28k (the form found in avian intestine) has been associated with lysosomes (25), the role of calbindin in this model is less clear. In the past, we have tested the facilitated diffusion model using cultures of the colonic carcinoma cell line Caco-2 (9, 11, 12, 32).

Caco-2 cells are a human colon adenocarcinoma cell line that can spontaneously differentiate in culture and acquire a small intestinal phenotype, e.g., they become polarized columnar epithelial cells, they form tight junctions and domes, and they express several markers that are unique to differentiated small intestinal epithelium (e.g., high sucrase-isomaltase mRNA and protein levels) (4, 31). The parental cell line of Caco-2 possesses at least two components necessary for vitamin D-regulated calcium transport in the facilitated diffusion model: calbindin D9k (11) and the vitamin D receptor (VDR) (13). In addition, they demonstrate saturable apical-to-basolateral calcium transport kinetics, net transport is positive in the apical-to-basolateral direction, and the rate of saturable transport can be increased by pretreatment with 1,25(OH)2D3 (12, 14). Vitamin D-induced upregulation of calcium transport requires transcriptional events (12) and is modulated by changes in the VDR content of the cell (32).

In contrast to these findings, Chirayath et al. (6) did not observe vitamin D-regulated calcium absorption using the AQ subclone of an established Caco-2 clone, Caco-2/15. Variation in the level of differentiation, i.e., expression of the small intestine enzyme markers su-
crase, alkaline phosphatase, and amino peptidase IV, has been well documented in clonal lines of Caco-2 (4), and this variability likely explains the difference between our data (12) and that of Chirayath et al. (6). However, while extreme variation between clones could render some of them useless for the study of calcium transport, we reasoned that this variability could also be utilized to test the details of the facilitated diffusion model of vitamin D-regulated calcium transport. Thus we propose that well-differentiating clones of Caco-2 (i.e., those that develop high expression of sucrase mRNA during spontaneous differentiation) will also have calcium transport characteristics more reflective of mature, midvillus small intestinal epithelial cells and that this will make them better, less variable models for the study of calcium absorption. In addition, identifying differences in calcium transport and vitamin D-induced gene expression between the parent line of Caco-2 and well-differentiating clones will permit us to test various features of the facilitated diffusion model.

MATERIALS AND METHODS

Supplies

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO); cell culture reagents, including fetal bovine serum (FBS), were obtained from BioWhittaker (Walkersville, MD), and cell culture plasticware was from Corning-Costar (Cambridge, MA).

Conditions of Cell Culture

The parental strain (HTB 37) and the BBe clone (CRL 2102) of the human colon adenocarcinoma cell line Caco-2 were obtained from American Type Culture Collection (Rockville, MD). The parental cells were studied between passages 24 and 50. BBe cells were described by Peterson and Mooseker (30), and they were studied between passages 52 and 77. TC7 cells, first described by Chantret et al. (4), were provided by Dr. Mark Failla (Ohio State Univ.) and studied between the 69th and 94th passages. The BBe and TC7 clones were selected for study because they have a more small intestine-like phenotype after they have differentiated in culture compared with the parental cell line (e.g., well-developed microvilli for BBe (30), high sucrase expression for TC7 (4)). All cells were routinely subcultured in 75-cm² plastic flasks and passaged when 75–85% confluent by dispersion in 0.1% trypsin. Cells were cultured in high-glucose (4.5 g/l) DMEM supplemented with 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 100 μM nonessential amino acids (Life Technologies, Rockville, MD), 50 μg/ml gentamycin (Life Technologies), 2 mM l-glutamine, 10 mM HEPES, and 20% (at seeding) or 10% (after confluence) FBS. Cell lines were maintained at 37°C in a 5% CO₂-95% air atmosphere.

To ensure that the cell lines would reach confluence concurrently (after 3–4 days in culture), different seeding densities were used for each cell line. For mRNA analysis, cells were seeded in six-well dishes (Becton Dickinson Labware, San Diego, CA) at 1.28 × 10⁶ (parental), 2.56 × 10⁶ (BBe), or 0.64 × 10⁶ cells/well (TC7). For VDR and calbindin D₉k protein analysis, cells were seeded in 75-cm² plastic flasks at 1 × 10⁶ (parental), 2 × 10⁶ (BBe), or 5 × 10⁵ cells/flask (TC7). For transepithelial calcium transport studies, cells were seeded on permeable membrane filter inserts (24.5-mm diameter, 0.4 μm) at 2 × 10⁵ (parental), 4 × 10⁵ (BBe), or 1 × 10⁵ cells/filter (TC7). Medium was changed every other day using DMEM + 10% FBS from days 2 through 10. Starting on day 11, media were changed daily.

Cell Treatments

To compare baseline and vitamin D-induced mRNA levels for the calcium transport channel CaT1 and epithelial calcium channel ECaC1, calbindin D₉k, the basolateral plasma membrane calcium ATPase isoform 1b (PMCA), and 1,25-hydroxyvitamin D₃ 24-hydroxylase (CYP24) and for net calcium transport among the three cell lines, cultures of cells were treated with either 10 nM 1,25-(OH)₂D₃ or vehicle (ethanol) for 8 h [for plasma membrane calcium ATPase (PMCA), CYP24, CaT1, ECaC1] or 48 h (for net calcium transport and calbindin D₉k). All treatment periods ended on day 15 of culture (11 days postconfluence). Treatments were diluted in DMEM + 5% FBS. Ethanol content in the control and treatments was 0.01%. There were no special treatments for the VDR protein or sucrase mRNA analysis, i.e., only basal levels at 11 days postconfluence were determined.

Calcium Transport Studies

All calcium transport studies were conducted on day 15 in culture (11 days postconfluence). Net transepithelial calcium transport was determined by subtracting the transport of calcium in the basolateral-to-apical direction from the transport of calcium in the apical-to-basolateral direction as described previously by Fleet and Wood (12). In addition, phenol red movement in each direction was assessed to determine differences in cell permeability between cell lines (12).

Western Blot Analysis of Nuclear VDR Protein Levels

After 15 days in culture, media were removed and cells were lysed using 1 ml TED buffer (10 mM Tris base, 1.5 mM EDTA, 1.6 mM dithiothreitol (DTT)) plus 10 mM sodium molybdate, 1.25 kallikrein inhibitory units (KIU/ml aprotnin, 300 μM phenylmethylsulfonyl fluoride (PMSP), and 1.6 μg/ml soybean trypsin inhibitor. Cell lysates were sonicated on ice (Fisher, model 50 sonic dismembrator) for 20-sec
bursts at the lowest setting. Sonicates were centrifuged (10,000 g, 4°C), and nuclear pellets were retrieved and washed three times with 400 μl TED buffer plus 0.5% Triton X-100 and 300 μM PMFS. After the third wash, each pellet was resuspended in 200 μl resuspension buffer (TED buffer plus 10 mM sodium molybdate, 12.5 KIU/ml aprotinin, and 300 μM PMFS).

The protein level of each nuclear pellet was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Twenty-microgram protein aliquots of each sample were solubilized in Laemmli buffer (20), denatured by heating at 95°C for 10 min, and cooled on ice for 5 min. Samples were separated by SDS-PAGE for 2 hr at 150 V using a 12% Tris-glycine gel (NOVEX, San Diego, CA). The samples were transferred from gels to Immobilon-P membranes (Millipore, Bedford, MA) for 2 hr at 200 mA (at 4°C), following the manufacturer’s instructions.

Western blot analysis was conducted by first incubating each membrane in 20 ml of membrane blocking solution (5% nonfat dried milk, 10% BSA, and 20 ml TBS-T buffer containing 20 mM Tris base, 500 mM NaCl, 3.8 mM HCl, 0.2% Tween-20, pH 7.6) for 1 hr at room temperature. After blocking, the membrane was washed five times using the TBS-T buffer. Afterward, the membrane was incubated in 20 ml primary blocking solution containing TBS-T buffer and 1 μg/ml unconjugated goat anti-rat IgG for 2 hr at room temperature. The membrane was then washed three times in TBS-T buffer and incubated overnight in 20 ml primary antibody solution (1:5,000 dilution of rat anti-chick VDR antibody 9A7 in TBS-T, Affinity Bioreagents, Golden, CO). The next day, the membrane was washed five times in 30 ml TBS-T buffer and then incubated in 20 ml of the secondary antibody solution (1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG in TBS-T). After incubation in the secondary antibody solution, the membrane was washed five times using 30 ml of TBS-T buffer. Specific binding was detected using the enhanced chemiluminescence Plus Western blotting detection reagents (Amer sham Pharmacia Biotech, Piscataway, NJ) as per the manufacturer’s instructions. Chemiluminescence was detected by exposure of the membranes to Hyperfilm (Amer sham), and the image was processed in an X-ray processor. Protein band intensities were recorded by densitometry using a Bio-Rad imaging densitometer and quantified using the Molecular Analyst program (version 1.5, Bio-Rad, Hercules, CA). After analysis of nuclear VDR (nVDR), the nVDR antibody was stripped from the blot by washing the membrane with 30 ml of TBS-T buffer. Specific binding was detected by exposure of the blot to X-ray film.

Cells containing the plasmid were isolated under ampicillin selection (50 μg/ml), and the plasmids with the insert in the correct orientation were identified by digesting with EcoRV and Scal. The cDNA insert was sequenced by a PCR-based method to verify the sequence. The recombinant protein was isolated from a culture of transformed E. coli using a nickel column to bind the COOH-terminal HIS tag following the procedure outlined by the manufacturer (Invitrogen).

Preparation of anti-human calbindin D9k antiserum. Polyclonal antibody against purified recombinant human calbindin D9k was prepared in rabbits by Q BIOgene (Carlsbad, CA).

Western blot analysis. After 15 days in culture, cells treated with vehicle or with 10 nM 1,25(OH)2D3 were scraped, harvested as pellets, and frozen at −80°C. Cell pellets were later resuspended in 1 ml homogenizing buffer (10 mM Tris-HCl, 5 mM DTT, 1 mM PMFS) per T-75 flask pellet and sonicated for 15 s at low setting on ice, and the sonicate was centrifuged (15 min, 10,000 g, 4°C) to remove cell debris. The supernatant was analyzed for protein content using a Bio-Rad protein assay. Fifty micrograms of total protein were denatured (5 min, 95°C) in Laemmli buffer, loaded onto 14% Tris-glycine gels (Invitrogen), and separated under denaturing conditions. Proteins were electroblotted onto Hy bond membranes (Amer sham). Western blot analysis was conducted using the WesternBreeze immunodetection kit (Invitrogen) following the manufacturer’s directions. A 1:1,000 dilution of the anti-human calbindin D9k antiserum was used as the primary antibody. Specific signal was detected by chemiluminescent detection using CDP-Star following the manufacturer’s directions (Roche Molecular Biochemicals, Indianapolis, IN). Specific proteins were visualized by exposure of the blot to X-ray film.

RNA isolation and RT-PCR Analysis

Cells were harvested into 1 ml of TriReagent per well of a six-well dish, and total cellular RNA was isolated using TriReagent as per the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). RNA was quantified using UV light spectrophotometry, and the integrity of the RNA was confirmed by gel electrophoresis (1.5% agarose).

RT-PCR was conducted as previously described by Fleet and Wood (12). Primer information and cycle conditions are 1 calbindin D9k (GenBank X65869), forward 5'-ATGATGACTA- AAAAACTCTCT-3', reverse 5'-CTGGGATATCCTTTTTTACTAA-3', 21–26 cycles, annealing temperature (Tm) = 55°C; 2 CYP24 (L13286), forward 5'-TCTCATGCTAAATACCCACG- GTG-3', reverse 5'-TCCGCTGCGAAGGGATGGG-3', 23 cycles, Tm = 55°C; 3 PMCA1 (J04027), forward 5'-AAA- CACAGATTGCAATGTGGAAT-3', reverse 5'-GGGATGA- GAGTTACGACTTT-3', 29 cycles, Tm = 55°C; 4 CaT1 (AF365927), forward 5'-TGATGGCGCTATCATGCGCCAG- C3', reverse 5'-GTAGAGCTGCTGTAGCTCTTG-3', 33 cycles, Tm = 61°C; 5 EcaC (AF304464), forward 5'-TGATGGCGCT- CACCAACACCAAT-3', reverse 5'-AGAATTGCCCCATG- GTTGGA-3', 40 cycles, Tm = 65°C; 6 sucrase-isomaltase (X63597), forward 5'-GGTGTCACATCATCAGTGCAG-3', reverse 5'-CCAGTTGATTTCTATGTTTCTCTT-3', 27 cycles, Tm = 55°C; 7 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AJ005371), forward 5'-CCATAGGCCAGCCTTCC-3', reverse 5'-AGTCACCCACGAGGATTG-3', 17 cycles, Tm = 55°C.

Because CaT1 and EcaC1 are closely related sequences, primers were designed to recognize sites with minimal homology between the two targets (primer 1, 48% mismatch; primer 2, 38% mismatch). Similarly, PMCA1 is part of a family of related genes; the primers were designed to regions...
of PMCA1 with minimal homology to the other family members.

The cycle numbers used with each primer set were designed to amplify the product within the linear range of amplification. PCR products were separated by agarose gel electrophoresis (100 V, 30 min) on 2.5% agarose gels containing ethidium bromide. PCR product levels were determined by densitometry on photographic negatives of the gels or by directly collecting gel data into the Bio-Rad FluorS imaging system. The expression of CYP24, calbindin D9k, PMCA, and CAT1 mRNA levels were normalized to the level of expression of the constitutively expressed control gene, GAPDH. CYP24 mRNA levels are not detectable under basal conditions. Thus GAPDH-corrected CYP24 mRNA levels were expressed relative to vitamin D-induced levels of the parental cells.

Statistical Analysis of Data

Data are reported as means ± SE. All experiments were repeated at three times for calcium transport and VDR analysis (n = 9 individual observations/treatment) and four times for mRNA analysis (n = 12). Treatment effects and interactions were determined by ANOVA with a P value < 0.05 used as the cut-off for statistical significance. When the plots of predicted values vs. residuals demonstrated that the data were not normally distributed, log transformations were conducted before statistical analysis. Pairwise comparisons were conducted using Fisher’s protected least significant difference test. All statistical analyses were conducted using the Systat statistical software package (SAS Institute, Cary, NC).

RESULTS

As expected, at 11 days postconfluent, mRNA levels of the small intestine differentiation marker sucrase-isomaltase were significantly higher in TC7 [29.4 ± 3.7 arbitrary units (AU)] and BBe cells (20.3 ± 2.9 AU) compared with the parental cells (5.0 ± 0.5 AU). At this time point, basal levels of VDR protein level in nuclear extracts was significantly higher in the TC7 and parental cultures compared with the BBe cells (Fig. 2, 1.33 ± 0.32 and 1.00 ± 0.17 vs. 0.38 ± 0.09 relative units, P = 0.04). TFIIb levels did not vary significantly between the three cell lines (Fig. 2A and data not shown).

To determine whether vitamin D-inducible events are dependent on variations in nVDR levels, we measured basal and vitamin D-induced levels of calcium transport and mRNA for proteins thought to be critical for regulated, transcellular calcium absorption in the intestine. Basal CYP24 mRNA level was not detectable in any of the three cell lines examined (data not shown). After 8 h of treatment with 10 nM 1,25(OH)2D3, CYP24 mRNA was significantly higher in TC7 and parental cells (1.15 ± 0.04 and 1.00 ± 0.07 AU, respectively) compared with BBe cells (0.64 ± 0.05, P < 0.001, Fig. 3A). Low, but detectable, levels of CaT1 mRNA were seen in each of the cell lines (average expression in all 3 lines = 0.17 ± 0.03 AU). After 1,25(OH)2D3 treatment (8 h, 10 nM), CaT1 mRNA level was increased in all three cell lines with the highest level being seen in BBe cells (2.91 ± 0.45 vs. 1.18 ± 0.20 AU for TC7, P = 0.01, and 1.41 ± 0.26 AU for parental, P = 0.067, Fig. 3B). In contrast, ECaC1 mRNA was not detectable in any of the cell lines although very low levels could be induced by vitamin D treatment in TC7 (e.g., requiring 40 cycles of PCR to observe; data not shown). Basal PMCA mRNA level was significantly lower in TC7 (0.65 ± 0.06 vs. 0.93 ± 0.15 and 0.84 ± 0.12 AU for parent and BBe, Fig. 3C). Calcitriol treatment increased PMCA mRNA levels to the greatest extent in BBe cells (102% increase, P < 0.001). PMCA mRNA level was not altered by calcitriol treatment in parental Caco-2 cells.

In absolute terms, the level of calbindin D9k mRNA was dramatically higher in TC7 cells (24.18 ± 2.63 AU) than in either BBe (0.81 ± 0.20 AU) or parental cells (0.297 ± 0.03 AU). Using Western blotting and an antibody raised against recombinant human calbindin D9k, we could detect calbindin D9k only in TC7 cells (Fig. 4B), reflecting the dramatic differences we noted in mRNA levels between the cell lines (TC7 30-fold higher than BBe, 90-fold higher than parental cells). Treatment with 10 nM 1,25(OH)2D3 for 48 h significantly increased calbindin D9k mRNA levels in all three cell lines (Fig. 4A). Expressed relative to each cell line’s own baseline, the increase in calbindin D9k mRNA levels was significantly higher in the BBe cells (3.57 ± 0.65 relative induction) compared with the parent (1.82 ± 0.13; P = 0.015) and TC7 cells (2.16 ± 0.15; P < 0.001, Fig. 4A). As with mRNA levels, vitamin D treatment increased the calbindin D9k protein levels in TC7 cells (Fig. 4B).

The final set of experiments was done to determine whether there were any differences in the basal or the
due to a high rate of basolateral-to-apical calcium lower net calcium transport we observed in TC7 was

nmol culture, cells were treated with 10 nM 1,25(OH)2D3 or vehicle for 8 h.

found that all three cell lines tested had a signiﬁcantly different from one another (Fisher’s protected LSD; P < 0.05). The lowest basal level of net calcium transport. Next, we tested LSD; their values were normalized for the expression of GAPDH mRNA within each sample. Because CYP24 mRNA is not detected in the absence of 1,25(OH)2D3 treatment, CYP24 mRNA data for BBe and TC7 are expressed relative to the induction seen in the parent cells (relative value = 1). Data represent means ± SE from 4 separate experiments performed in triplicate (n = 12 observations). Bars with different letters are signiﬁcantly different from one another (Fisher’s protected LSD; P < 0.05).

Fig. 3. Induction of CYP24, CaT1, and PMCA mRNA levels by 1,25(OH)2D3 in parent, BBe, and TC7 cells. On the 15th day in culture, cells were treated with 10 nM 1,25(OH)2D3 or vehicle for 8 h. RNA was isolated and analyzed for CYP24 (A), CaT1 (B), or PMCA mRNA levels (C) by RT-PCR. PCR bands were quantiﬁed by densitometry, and mRNA values were normalized for the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA within each sample. Because CYP24 mRNA is not detected in the absence of 1,25(OH)2D3 treatment, CYP24 mRNA data for BBe and TC7 are expressed relative to the induction seen in the parent cells (relative value = 1). Data represent means ± SE from 4 separate experiments performed in triplicate (n = 12 observations). Bars with different letters are signiﬁcantly different from one another (Fisher’s protected LSD; P < 0.05).

B vitamin D-induced levels of calcium transport among the three cell lines. Table 1 and Fig. 5 show several important ﬁndings regarding calcium transport in these cells. First, we found that the cell with the highest basal level of calbindin D9k (TC7) had the lowest basal level of net calcium transport. Next, we found that all three cell lines tested had a signiﬁcant increase in net calcium transport after 48 h of treatment with 10 nM 1,25(OH)2D3. Finally, we saw that the change in calcium transport after vitamin D treatment was signiﬁcantly greater in BBe cells compared with TC7 or parental cells (0.39 ± 0.03 vs. 0.13 ± 0.05 nmol·well−1·min−1; P < 0.001, and 0.20 ± 0.02 nmol·well−1·min−1, P < 0.001, respectively). The lower net calcium transport we observed in TC7 was due to a high rate of basolateral-to-apical calcium transport rather than lower apical-to-basolateral movement (Table 1).

Table 1. Induction of unidirectional and net calcium transport across monolayers of parent, TC7, and BBe Caco-2 cells by 1,25(OH)2D3 (10 nM, 48 h)

<table>
<thead>
<tr>
<th>Cell/Treatment</th>
<th>Change A to B</th>
<th>Change B to A</th>
</tr>
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<tbody>
<tr>
<td>Parent Control</td>
<td>0.52 ± 0.05b</td>
<td>0.33 ± 0.03c</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.85 ± 0.04c</td>
<td>0.53 ± 0.02d</td>
</tr>
<tr>
<td>Change</td>
<td>0.34 ± 0.05</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>BBe Control</td>
<td>0.29 ± 0.02a</td>
<td>0.16 ± 0.01a</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.80 ± 0.04c</td>
<td>0.28 ± 0.02b</td>
</tr>
<tr>
<td>Change</td>
<td>0.51 ± 0.03c</td>
<td>0.12 ± 0.01a</td>
</tr>
<tr>
<td>TC7 Control</td>
<td>0.24 ± 0.02a</td>
<td>0.23 ± 0.01ab</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.65 ± 0.04b</td>
<td>0.44 ± 0.03d</td>
</tr>
<tr>
<td>Change</td>
<td>0.41 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
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</table>

Values represent means ± SE from 3 independent experiments (n = 9 observations/treatment. 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; A-to-B, apical to basolateral; B to A, basolateral to apical. *Values for change in transport due to vitamin D treatment signiﬁcantly different from both the parent and TC7 level. Values within a column with different letter superscripts are signiﬁcantly different from one another [Fisher’s protected least signiﬁcant difference test (LSD); P < 0.05].
in the enterocyte. Low VDR level has been proposed to nVDR is a critical determinant of vitamin D responses cells (32), have suggested that the cellular level of the addition, some studies, including our own in Caco-2 of the intracellular calcium ferry, calbindin D9k. In this process was the vitamin D-dependent production of 1,25(OH)2D3 in parent, BBe, and TC7 cells. Cells grown on perme - to 48 h
starting on the 13th day in culture. On day 15 in culture, unidirectional calcium transport was determined in the apical-to-basolateral and the basolateral-to-apical direction using parallel cultures. Net transport was determined by subtracting the basolateral-to-apical movement from the apical-to-basolateral movement. Bars represent means ± SE from 3 independent experiments (n = 9 observations/treatment). Bars with different letters are significantly different from one another (Fisher’s protected LSD; P < 0.05). @Significantly different from parent and TC7 level.

Fig. 5. Induction of net apical-to-basolateral calcium transport by 1,25(OH)2D3 in parent, BBe, and TC7 cells. Cells grown on permeable filter supports were treated with 10 nM 1,25(OH)2D3 for 48 h

DISCUSSION
A large amount of association data from animal experiments supports the facilitated diffusion model of intestinal calcium absorption. In this model (summarized in Fig. 1), calcium enters the absorptive enterocyte down an electrochemical gradient through a brush-border membrane calcium channel (presumably CaT1 or ECaC1), diffuses across the cell with the aid of the calcium binding protein calbindin D9k, and is then extruded by an active, energy-requiring step at the basolateral membrane by a plasma membrane calcium ATPase, PMCA1b. Calcium absorption efficiency is increased by 1,25(OH)2D3. Based on a mathematical evaluation of available protein and transport data, Bronner et al. (2) argued that the rate-limiting step in this process was the vitamin D-dependent production of the intracellular calcifer, calbindin D9k. In addition, some studies, including our own in Caco-2 cells (32), have suggested that the cellular level of the nVDR is a critical determinant of vitamin D responses in the enterocyte. Low VDR level has been proposed to explain age-associated intestinal resistance to vitamin D (7, 17), which in turn could be responsible for the low calbindin D9k levels and reduced calcium absorption seen with aging (10, 36). Based on this idea, and our previous demonstration of the importance of VDR level in Caco-2 cells, we hypothesized that 1) the cell line with the lowest VDR content would have the lowest responsiveness to 1,25(OH)2D3 treatment and 2) the cell line with the highest calbindin D9k content would have the highest rate of transcellular calcium transport, regardless of whether the cells were treated with 1,25(OH)2D3. However, our data do not support these theories.

Low VDR content in BBe cells was associated with the lowest CYP24 mRNA induction by 1,25(OH)2D3. The positive relationship between CYP24 induction and VDR content is consistent with our previous observations in Caco-2 cells stably transfected with an inducible VDR transgene (32) and with reports from others in osteoblasts treated with dexamethasone or parathyroid hormone (5, 18). In contrast, we found that vitamin D-induced net apical-to-basolateral calcium transport and CaT1, PMCA, and calbindin D9k mRNA levels were all highest in BBe. We hypothesize that this difference is due to the less robust induction of CYP24 in BBe cells. CYP24 is the first step in the metabolic inactivation of 1,25(OH)2D3 (21, 34), and thus cells with a greater capability to induce CYP24 may have reduced cellular levels of 1,25(OH)2D3; this would give the appearance of reduced intestinal activity of the hormone (e.g., Figs. 3, B and C, and 4A and Table 1). Further studies are required to test this hypothesis.

Another interesting finding from our study is that high calbindin D9k levels, as we observed in TC7 cells (mRNA levels 29 times greater than BBe and 86 times greater than parental), do not ensure high rates of calcium transport in Caco-2 cells (Fig. 5, Table 1). This observation is consistent with those made in chicks and rats. Spencer et al. (33) showed that after 1,25(OH)2D3 injection in rachitic chicks, both calcium absorption and calbindin D28k proteins were elevated. However, while calbindin D28k remained elevated for 32 h after its peak induction, a high rate of calcium absorption was not maintained during that same period, falling to 25% of its maximum rate. Using di- and trihomoanalogues of 1,25(OH)2D3, Krisinger et al. (19) increased intestinal calbindin D9k mRNA without concomitant

Table 2. Regulation of phenol red transport by 1,25(OH)2D3 in parent, TC7, and BBe Caco-2 cells

<table>
<thead>
<tr>
<th>Phenol Red Transport, nmol well−1 min−1</th>
<th>Baseline</th>
<th>1,25 (OH)2 D3 (10 nM, 48 h)</th>
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<tr>
<td></td>
<td></td>
<td>Apical to basolateral</td>
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<tr>
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<tr>
<td></td>
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<td>Basolateral to apical</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parent</td>
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Values are means ± SE from 3 independent experiments (n = 9 observations/treatment). Values within a column with different letter superscripts are significantly different from one another (Fisher’s protected LSD; P < 0.05).
elevations in calcium absorption. Finally, Wang et al. (35) found that accumulation of calbindin D9k mRNA and protein did not lead to higher intestinal calcium absorption in rats injected with 1,25,28-trihydroxyvitamin D3. These studies, and our current findings, cast suspicion on the hypothesis that calbindin D9k is the rate-limiting determinant of calcium absorption. However, while our data do not support a role for calbindin D9k as the rate-limiting determinant for transcellular calcium transport in Caco-2 cells, our data do support the use of relative calbindin D9k mRNA induction as a marker for vitamin D-mediated absorptive capacity: BBe cells have the highest relative induction and the highest induction of net transport.

The proteins mediating calcium uptake into enterocytes during calcium absorption have been unknown until recently. In 1999, Hoenderup et al. (16) and Peng et al. (28) identified two closely related calcium channels from mammalian intestine, ECaC and CaT1, respectively. ECaC mRNA is reported to be highly expressed in human small intestine, kidney, and pancreas but less strongly expressed in tissues like distal colon (22). It has an apical localization and is coexpressed with calbindin D9k and PMCA in rabbit intestinal cells (15). CaT1 mRNA is present throughout the gastrointestinal tract as well as in other tissues, e.g., pancreas, prostate, liver, kidney, and testis (29). Both groups have proposed that their channel is responsible for uptake of Ca into the absorptive epithelial cell; it is possible that these two channels are functionally redundant and that either channel, or other closely related proteins, can mediate apical calcium entry during intestinal calcium absorption.

None of these published reports provide evidence to support the regulation of either ECaC or CaT1 by 1,25(OH)2D3. However, unpublished data from our lab show that intestinal CaT1 mRNA levels are reduced in VDR knockout mice and in normal mice fed a high-calcium diet to drive down serum 1,25(OH)2D3 levels. In addition, we have previously reported that 1,25(OH)2D3 treatment causes a rapid and marked increase in CaT1 mRNA that precedes the induction of calbindin D9k in Caco-2 cells (37). These observations are consistent with the hypothesis that CaT1 serves as a gatekeeper for calcium absorption. Our current data show that both CaT1 (all 3 cell lines) and ECaC (TC7 only) mRNA levels are upregulated by treatment of Caco-2 cells with 10 nM 1,25(OH)2D3 for 8 h (Fig. 3B and data not shown). However, the fact that we detected little (TC7) or no (BBe and parental) expression of ECaC in any of the cell lines suggests that ECaC is not necessary for net apical-to-basolateral calcium transport in Caco-2 cells. The vitamin D-induced increase in CaT1 mRNA levels was associated with the hormone-dependent increase in net apical-to-basolateral calcium transport. This suggests that induction of CaT1 production may be important for the adaptive upregulation of calcium transport in these cells. This is in conflict with the elegant studies of Chandra et al. (3). Using ion microscopy on intestinal sections from rachitic and vitamin D-replete chicks, they found that while calcium absorp-

REFERENCES


