Specific 1,25(OH)$_2$D$_3$-mediated regulation of transcellular calcium transport in Caco-2 cells

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Fleet, James C., and Richard J. Wood. Specific 1,25(OH)$_2$D$_3$-mediated regulation of transcellular calcium transport in Caco-2 cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G958–G964, 1999.—Calcium transport in the apical-to-basolateral (A-to-B) or B-to-A direction was examined in cells treated with 10 nM 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$, calcitriol] for up to 72 h. Net A-to-B calcium transport was positive at all time points and increased from 0.14 ± 0.06 to 0.50 ± 0.01 nmol·well$^{-1}$·min$^{-1}$ after 72 h of calcitriol treatment. Neither phenol red transport nor transepithelial electrical resistance was altered by calcitriol treatment, suggesting that the increase in net A-to-B calcium transport was not due to paracellular movement. Neither 25-hydroxyvitamin D$_3$ nor 24,25-dihydroxyvitamin D$_3$ (100 nM, 48 h) alters basal or calcitriol-stimulated A-to-B calcium transport. Treatment with the calmodulin antagonist trifluoperazine (50 µM) reduced calcitriol-stimulated A-to-B calcium transport by 56%. The transcription inhibitor actinomycin D inhibited calcitriol-regulated A-to-B calcium transport as well as calbindin D$_{9k}$ and 24-hydroxylase mRNA accumulation. These data demonstrate that calcitriol-mediated A-to-B calcium transport in Caco-2 cells is a specific, transcellular process that requires transcriptional events normally mediated through the vitamin D receptor.

trifluoperazine; 24-hydroxylase; calbindin D$_{9k}$

DYNAMICS OF INTESTINAL calcium absorption have been characterized in a wide variety of species and systems (32), and we have understood for over 50 years that the efficiency of intestinal calcium absorption is related to vitamin D status (31). However, the observation that the efficiency of calcium absorption declines with aging in animals and humans (2, 10) and our limited understanding of the molecular details of intestinal calcium absorption justify the continued mechanistic evaluation of this process. The active hormonal form of vitamin D$_3$, 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$, calcitriol], is thought to regulate intestinal calcium absorption by increasing the transcellular flux of calcium through enterocytes (28). Two models have been proposed to describe the mechanism of vitamin D-mediated calcium absorption. In the facilitated diffusion model 1,25(OH)$_2$D$_3$ stimulates calcium movement at three important steps: 1) entry of calcium into the cell via a putative apical membrane transport protein or channel, 2) intracellular diffusion of calcium through the cytosol via the ferrying action of calbindin D, and 3) extrusion across the basolateral membrane via an ATP-dependent calcium pump (8). In the vesicular transport model calcium enters the cell either through a calcium channel or by endocytosis and is localized into lysosomes; calcium from lysosomes is thought to be extruded from cells by exocytosis (29). Some evidence suggests that calbindin D is also involved in this process (30). Although evidence from various experimental approaches exists to support both of these models of vitamin D-mediated intestinal calcium transport, the development of a cell culture model for examining the molecular details of 1,25(OH)$_2$D$_3$-mediated calcium absorption would be useful for testing details of each model. With this in mind, our group has previously reported on several key characteristics of calcium transport in Caco-2 cells, a human colon adenocarcinoma cell line (17, 20, 24).

In culture, Caco-2 cells spontaneously differentiate and form a polarized epithelial monolayer with tight junctions and express a differentiated cell phenotype consistent with absorptive small intestine-like enterocytes (33, 36). Since its initial characterization as a model for drug transport, Caco-2 cells have been used as an in vitro model to study intestinal transport of micronutrients, including minerals such as zinc (19, 34), iron (1, 3, 25), and calcium (17, 18, 20, 23, 24). In particular, findings from our laboratory show how various characteristics of Caco-2 cells make them a potentially useful model to study vitamin D-induced calcium transport. For example, Caco-2 cells have a functional vitamin D receptor (23) and have calcium transport kinetics that suggest the presence of both a saturable and nonsaturable calcium transport pathway, similar to what has been observed in human and animal intestine. 1,25(OH)$_2$D$_3$ treatment induces the saturable, but not diffusional, component of calcium transport (24) and induces accumulation of calbindin D$_{9k}$ and 24-hydroxylase mRNA (17, 20).

Two studies were recently published that suggest that calcium transport across monolayers of Caco-2 cells is purely diffusional, i.e., nonspecific (7, 12). In a study by Blais et al. (7) no attempt was made to investigate vitamin D-mediated calcium transport, whereas a study by Chirayath et al. (12) found that in a rapidly proliferating, less differentiated clone of Caco-2 cells (AQ), 1,25(OH)$_2$D$_3$ reduced transepithelial electrical resistance (TEER), thus permitting an increased paracellular movement of calcium. In contrast, we now report additional evidence to demonstrate that the regulation of calcium transport in the parent cell line of
Caco-2 cells is a transcellular pathway and is due to the specific regulation of cellular events by 1,25(OH)2D3.

MATERIALS AND METHODS

Conditions of cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD) were propagated and maintained in high-glucose DMEM (4.5 g glucose/l), supplemented with 10 mM HEPES, 44 mM sodium bicarbonate, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 50 

µg/ml gentamicin sulfate, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, final pH 7.2. For routine passage, medium contained 20% fetal bovine serum (FBS), cells were seeded into T75 flasks at a density of 1 × 106 cells/flask, and cells were passaged every 3–4 days when the cultures were 80% confluent. For transport studies, 250,000 cells were seeded onto permeable membrane filter inserts (24.5-mm diam, 0.4 µm; Costar, Cambridge, MA). For mRNA studies, 200,000 cells were seeded into six-well dishes (35-mm diam, Costar). Cultures were used for experiments between passage 25 and 60. After cells reached confluence they were fed with DMEM supplemented with 10% FBS every other day until day 12 in culture (the point of maximum expression of enzymatic markers of differentiation (23)). Cells were subsequently fed every day until the end of the experiment (day 15 in culture). Cell culture medium, nutrients, and antibiotics were purchased from BioWhittaker (Walkersville, MD), FBS was purchased from HyClone Laboratories (Logan, UT), vitamin D compounds were obtained from Bio-Mol (Plymouth Meeting, PA), and chemicals for all other purposes were obtained from Sigma Chemical (St. Louis, MO).

Cell treatments. In the first calcium transport experiment, cells were treated with 10 nM 1,25(OH)2D3 for 0, 24, 48, or 72 h, and calcium transport was determined in the apical-to-basolateral (A-to-B) and B-to-A direction. In all experiments, treatments were timed so that cell treatment ended on day 15 in culture. In the second experiment, cells were treated with vehicle, 10 nM 1,25(OH)2D3, 100 nM 25-hydroxyvitamin D3 [25(OH)2D3], or 100 nM 24,25-dihydroxyvitamin D3 [24,25(OH)2D3] for 48 h. The 25(OH)D3 and 24,25(OH)2D3 treatments were also combined with the 10 nM 1,25(OH)2D3 treatment to determine if these compounds altered the cellular response to calcitriol. In the third experiment, vehicle or 1,25(OH)2D3 (10 nM, 48 h) were also treated with trifluoperazine (TFP, 0–50 µM) during the transport study that includes a 30-min equilibration period followed by a 30-min transport period. Finally, to assess the importance of transcriptional events on vitamin D-inducible calcium transport, cells were treated with 1 µg/ml actinomycin D for 8 h followed by a cocubation with 1,25(OH)2D3 or ethanol vehicle for 40 h. Calcium transport was studied after this period. The effect of 4 µg/ml actinomycin D or 10 µg/ml cyclohexamide on 24-hydroxylase and calbindin D9k mRNA levels was assessed after an initial 1-h preincubation with the inhibitor, followed by treatment with 1,25(OH)2D3 for either 8 h (for 24-hydroxylase mRNA) or 48 h (calbindin D9k) in the presence or absence of the inhibitor. Specific mRNA levels were determined by RT-PCR. In all experiments inhibitors and vitamin D compounds were diluted in DMEM plus 5% FBS. Control treatments were comprised of vehicles for the inhibitors or vitamin D compounds diluted to the same extent as the most concentrated inhibitor or vitamin D stock used in a particular experiment (0.1% or less final ethanol concentration).

Calcium transport studies. Transport studies in the A-to-B direction were conducted as previously reported by Fleet and Wood (20). Unless noted otherwise, calcium transport refers to transport in the A-to-B direction. The transport buffer contained 1 µCi 45Ca/ml and 500 µM calcium chloride. In all experiments, phenol red was included in the transport buffer at 500 µM as a means of measuring diffusional transport through the monolayer (9). The percentage of phenol red transported per minute was calculated, and the equivalent amount of calcium was subtracted from the value for total calcium transport to derive the value for saturable calcium transport as described previously (20). Experiments had six wells per treatment and were conducted three times.

Effect of 1,25(OH)2D3 treatment on TEER. Electrical resistance of the Caco-2 cell monolayer grown on permeable membrane filter supports was measured using a voltohmmeter (World Precision Instruments, Sarasota, FL). Resistance was measured from day 3 in culture (just before confluence) to day 15 in culture. TEER was measured in six wells at each of the time points. This experiment was repeated three times. In addition, the effect of treating 12-day-old cultures for 72 h with 10 nM 1,25(OH)2D3 on TEER was examined (n = 6 wells/treatment, repeated 8 times).

RT-PCR analysis for mRNA levels. After experimental treatments, cells were harvested and RNA was isolated and analyzed for 24-hydroxylase, calbindin D9k, and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels by semiquantitative RT-PCR using conditions previously described (17, 19). Primer sets were derived from previously published sequences and are GAPDH, forward primer bases 386–403 and reverse primer bases 561–580; calbindin D9k, forward primer bases 1–18 and reverse primer bases 218–237; 24-hydroxylase, forward primer bases 1633–1653 and reverse primer 1912–1932 (11). To minimize the potential for variability in the reverse transcriptase reaction, cDNA was prepared from total cellular RNA for all samples at the same time, using the same reagents. Specific target messages were detected by amplifying cDNAs for 20 (GAPDH), 22 (24-hydroxylase), or 25 cycles (calbindin D9k). These cycles were previously determined to fall within linear range of amplification efficiency for each of the primer sets. Each target message was detected in a separate PCR reaction. The identity of the three PCR products was confirmed by subcloning the PCR product into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) followed by sequencing (data not shown). PCR products were subjected to 2.5% agarose gel electrophoresis followed by visualization of ethidium bromide-stained gels under ultraviolet light. PCR product levels were determined by densitometry on photographic negatives of the gels. Data were normalized to the level of GAPDH expression within the sample and then expressed relative to the expression seen in control (calbindin D9k) or vitamin D (24-hydroxylase)-treated cells.

Analysis of data. Data were analyzed by ANOVA using the Systat statistical package (35). Multiple comparisons were done using Fisher’s protected least-significant difference.
RESULTS

We utilized two approaches to assess the impact of 1,25(OH)2D3 on the permeability of the Caco-2 monolayer: 1) TEER and 2) phenol red transport. TEER increased as the cells in the monolayer reached full differentiation over 15 days in culture (Fig. 1). However, treatment of 12-day-old cultures with 10 nM 1,25(OH)2D3 for 72 h did not significantly reduce TEER [control, 1,044.0 ± 30.8 Ω·cm2; 1,25(OH)2D3, 985.5 ± 28.8 Ω·cm2]. The level of phenol red movement across the monolayer was 0.5% per hour in both A-to-B and B-to-A directions and was not altered by time of vitamin D treatment. Phenol red transport was not increased by calcitriol treatment in any of the other experiments presented (see Fig. 4 and Table 1).

Our initial calcium transport experiment examined whether there was net A-to-B calcium transport across a monolayer of fully differentiated Caco-2 cells. As shown in Fig. 2, total calcium transport in the A-to-B direction was progressively elevated by increasing time of treatment with 10 nM 1,25(OH)2D3 so that by 72 h transport it had increased 2.1-fold to 1.006 ± 0.008 nmol·well−1·min−1 (Fig. 2). Initially, B-to-A movement was 2.7% per hour or 70% of the A-to-B value. However, unlike A-to-B transport, B-to-A transport was increased by calcitriol treatment only after the initial 24-h period (from 0.337 ± 0.017 to 0.466 ± 0.008 nmol·well−1·min−1) and did not change significantly thereafter. As a result, net calcium flux (A-to-B minus B-to-A) increased 3.5-fold, from 0.14 ± 0.058 to 0.495 ± 0.013 nmol·well−1·min−1 with 72 h of 1,25(OH)2D3 treatment (Fig. 2).

In our next experiment, we examined whether other vitamin D compounds that were chemically related to 1,25(OH)2D3 could stimulate calcium transport in Caco-2 cells. Neither exposure with 25(OH)D3 nor 24,25(OH)2D3 (100 nM, 48 h) increased total A-to-B calcium movement, whereas 10 nM 1,25(OH)2D3 for 48 h increased transport by 56% (Table 1). Combining 25(OH)D3 or 24,25(OH)2D3 with 1,25(OH)2D3 did not alter the induction of calcium transport by 1,25(OH)2D3.

If 1,25(OH)2D3 were to stimulate transcellular calcium transport, we hypothesized that inhibition of calmodulin-dependent events (e.g., brush-border calcium uptake and basolateral membrane calcium extrusion) should limit total A-to-B calcium transport across the Caco-2 monolayer. Figure 3 shows that increasing amounts of the calmodulin antagonist TFP progressively inhibited the vitamin D-stimulated portion of calcium transport. The maximal inhibition of 56% was observed at 50 µM. Similar findings were seen in an experiment that examined only the effect of 50 µM TFP on A-to-B calcium transport and phenol red movement (data not shown). In this experiment calcitriol-mediated A-to-B calcium transport was blocked by TFP, whereas phenol red movement was increased from 1.0 ± 0.05 to 1.7 ± 0.05% Thus even as diffusion through the monolayer was increased, the effect of calcitriol on calcium transport was blocked.

In our final set of experiments, we examined the effect of the transcription inhibitor actinomycin D on the 1,25(OH)2D3-mediated component of calcium transport. Actinomycin D increased total A-to-B calcium movement across the monolayer and eliminated the difference in A-to-B calcium transport between control and vitamin D-treated cells. The increase in total calcium transport was due to enhanced paracellular diffusion, as demonstrated by increased phenol red movement from 0.59 to 1.75% per hour. However, when A-to-B calcium transport was corrected to account for this increase in diffusional flux, actinomycin D prevented the 1,25(OH)2D3-mediated increase in transcellular calcium transport (Fig. 4). This coincided with an inhibition of 1,25(OH)2D3-mediated gene expression as
1,25(OH)2D3 treatment (10 nM, 72 h) did not increase calcium transport and changes in TEER. These observations are consistent with our previous reports showing that 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3, 24,25(OH)2D3, 24,25-dihydroxyvitamin D3. Treatments were given for 48 h before measurement of calcium transport. Values with different superscripts are significantly different (P < 0.05).

Values are means ± SE; n = 6 for all treatments but control and calcitriol alone (n = 12). Saturable transport is total calcium transport corrected for diffusional (phenol red) transport. 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3, 24,25(OH)2D3, 24,25-dihydroxyvitamin D3. Treatments were given for 48 h before measurement of calcium transport. Values with different superscripts are significantly different.

Another observation that shows vitamin D-inducible A-to-B calcium transport is transcellular in Caco-2 cells is that TFP suppressed the vitamin D-mediated component of calcium transport. 1,25(OH)2D3- and actinomycin D-mediated calcium transport in Caco-2 cells reflects a movement through a transcellular pathway. Our TFP data in Caco-2 cell monolayers is similar to the effect that Favus et al. (16) observed for TFP on vitamin D-mediated calcium absorption in rat intestine.

Finally, the transcription inhibitor actinomycin D blocked vitamin D-mediated A-to-B calcium transport (Fig. 4). This suggests that de novo RNA synthesis is required for 1,25(OH)2D3-mediated intestinal calcium transport. Our data are in contrast to findings by Bikle et al. (6), who showed that treating rachitic chicks with actinomycin D inhibited vitamin D-mediated intestinal calcium absorption in rat intestine.

Table 1. Regulation of apical-to-basolateral calcium transport by vitamin D metabolites

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Calcium Transport</th>
<th>Saturable Calcium Transport</th>
<th>Phenol Red Transport</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nmol·well⁻¹·min⁻¹</td>
<td>%/h</td>
<td>nmol·well⁻¹·min⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>0.25 ± 0.01⁺</td>
<td>2.02 ± 0.08⁺</td>
<td>0.23 ± 0.01⁺</td>
</tr>
<tr>
<td>1,25(OH)2D3 (10 nM)</td>
<td>0.39 ± 0.02⁻</td>
<td>3.09 ± 0.18⁻</td>
<td>0.37 ± 0.02⁻</td>
</tr>
<tr>
<td>25(OH)D3 (100 nM)</td>
<td>0.22 ± 0.01⁻</td>
<td>1.73 ± 0.08⁻</td>
<td>0.20 ± 0.01⁻</td>
</tr>
<tr>
<td>1,25(OH)2D3 + 25(OH)D3</td>
<td>0.43 ± 0.01⁰</td>
<td>3.41 ± 0.23⁰</td>
<td>0.41 ± 0.03⁰</td>
</tr>
<tr>
<td>24,25(OH)2D3 (100 nM)</td>
<td>0.25 ± 0.01⁺</td>
<td>1.97 ± 0.10⁺</td>
<td>0.22 ± 0.01⁺</td>
</tr>
<tr>
<td>1,25(OH)2D3 + 24,25(OH)2D3</td>
<td>0.42 ± 0.02⁰</td>
<td>3.39 ± 0.12⁰</td>
<td>0.39 ± 0.01 khủng</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for all treatments but control and calcitriol alone (n = 12). Saturable transport is total calcium transport corrected for diffusional (phenol red) transport. 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3, 24,25(OH)2D3, 24,25-dihydroxyvitamin D3. Treatments were given for 48 h before measurement of calcium transport. Values with different superscripts are significantly different.

Fig. 3. Calmodulin inhibitor trifluoperazine (TFP) reduces 1,25(OH)2D3 inducible transcellular calcium transport. Cultures of Caco-2 cells were grown on permeable membrane filter supports for 13 days and then treated for 48 h with 10 nM 1,25(OH)2D3 or ethanol control. For 30-min before measurement of calcium transport and during 30-min transport study, cells were treated with 0–50 µM TFP. Calcium transport in apical-to-basolateral direction was determined as described in MATERIALS AND METHODS. Values are means ± SE of 3 well/time point. Bars with common letter are not significantly different.

Fig. 4. Actinomycin D inhibits 1,25(OH)2D3-mediated transcellular calcium transport. Cultures of Caco-2 cells were grown on permeable membrane filter supports for 13 days. After 8-h pretreatment with 1 µg/ml actinomycin D, cells were treated for 40 h with 10 nM 1,25(OH)2D3 or ethanol control in presence or absence of 1 µg/ml actinomycin D. Calcium and phenol red transport in apical-to-basolateral direction were determined as described in MATERIALS AND METHODS. Saturable transport was calculated as difference between total calcium transport and percent of transport represented by phenol red movement across monolayer. Values are means ± SE of 6 well/time point. Superscripts refer to level of saturable transport resulting from treatment. Bars with a common letter are not significantly different.
cycloheximide or actinomycin D blocked the production of several 1,25(OH)2D3-inducible proteins in the intestine but did not block 1,25(OH)2D3-stimulated calcium absorption. Although our observations could be due to the prolonged treatment of actinomycin D we used in our study (48 h), a shorter 24-h protocol yielded similar results (data not shown). We have previously noted that 12–16 h of vitamin D treatment are needed to significantly alter calcium transport in Caco-2 cells (20). This suggests the Caco-2 cell system may be different from the rachitic chick and that it adapts to our study (48 h), a shorter 24-h protocol yielded similar results. Although our observations could be due to the prolonged treatment of actinomycin D we used in our study (48 h), a shorter 24-h protocol yielded similar results (data not shown). We have previously noted that 12–16 h of vitamin D treatment are needed to significantly alter calcium transport in Caco-2 cells (20). This suggests the Caco-2 cell system may be different from the rachitic chick and that it adapts to the vitamin D treatment group (24-hydroxylase).

Calbindin D9k is a small-molecular-weight calcium-binding protein proposed to function as either an intracellular calcium buffer or an intracellular ferry protein that facilitates diffusion of calcium across the cell (8, 13). Actinomycin D treatment completely blocked vitamin D-induced accumulation of calbindin D9k mRNA in the Caco-2 cells; thus the inhibition in A-to-B calcium transport might be due to blocking production of this protein. Inhibition of protein translation with cycloheximide also blocked the 1,25(OH)2D3-induced accumulation of calbindin D9k mRNA, but not 24-hydroxylase mRNA, in Caco-2 cells (Fig. 5). This is consistent with previous work that demonstrates posttranscriptional regulation of calbindin D9k message in rat intestine and suggests that vitamin D could induce the synthesis of a protein that stabilizes the calbindin D9k mRNA. Further research is being conducted to more fully understand the mechanism by which vitamin D results in calbindin D9k mRNA accumulation.

Although our observations are consistent with the presence of a transcellular pathway for calcium transport in Caco-2 cells, two recent studies contradict this notion. Blais et al. (7) found that basal A-to-B calcium transport across Caco-2 monolayers (used between passage 25 and 40) was equal to paracellular transport (assessed with mannitol) at approximately 3% per hour. However, the values reported for mannitol transport by Blais et al. (7) were significantly higher than the 0.5% per hour previously reported in Caco-2 cells by Han et al. (25) and Chirayath et al. (12). This suggests that the integrity of their Caco-2 monolayers used by Blais et al. (7) may have been impaired or altered in some way so as to make them unacceptably leaky for calcium transport studies. In addition, these investigators did not examine the influence of 1,25(OH)2D3 on transport, thus their studies do not specifically address the suitability of the Caco-2 model to study the mechanism of vitamin D-regulated intestinal calcium absorption.

Chirayath et al. (12) have reported data using Caco-2 cells that directly conflict with several of our critical findings. For example, they found that 10 nM 1,25(OH)2D3 treatment for up to 72 h reduced TEER in Caco-2 monolayers. In addition, they did not observe a net A-to-B flux of calcium under basal or 1,25(OH)2D3-stimulated conditions nor did they see a reduction in 1,25(OH)2D3-inducible calcium transport following treatment with the calcium pump inhibitor calmidazolium. We believe that these conflicting observations may be due to major differences in the characteristics of the Caco-2 cell population examined in the two studies. First, Chirayath et al. (12) used a subclone of Caco-2 cells (AQ) derived from an established clone (Caco-2/15) after passage 100. Yu et al. (37) showed that high passage number Caco-2 cells (93–108) have lower carrier-mediated transport, higher TEER, and lower alkaline phosphatase activity (a marker of intestinal cell differentiation) than low passage number cells (28–36 passages). They suggest that this was due to selection of fast-growing subpopulations from the original heterogeneous Caco-2 cell line during repeated passaging. Features of a less-differentiated cell phenotype are also seen in the AQ subclone used by Chirayath et al., e.g., reduced doubling time (24 vs. 36 h) and lower alkaline phosphatase activity (60 vs. 190 mU/mg protein), compared with the Caco-2/15 parent clone and TEER values 50% higher than those we report for the parent stock. These differences in the AQ subclone show how various clonal cell lines of Caco-2 can have important phenotypic differences, such as a reduced ability to fully differentiate, that could render them inappropriate as an experimental model to study vitamin D-mediated calcium transport.
Next, we felt it was noteworthy that Chirayath et al. (12) did not see an inhibition of vitamin D-mediated A-to-B calcium transport after treatment with 100 µmol/l calmidazolium. However, Gietzen et al. (22) previously showed and we confirmed (data not shown) that the maximum concentration of calmidazolium (also called R-24571) in aqueous medium at pH 7.0 is 20 µM. As a result, we believe there is reason to question the validity of the calmidazolium data presented by Chirayath et al. (12) as well as their conclusion that calcium is not transported by a transcellular pathway in Caco-2 cells.

In summary, we have shown that the treatment of Caco-2 cells with 1,25(OH)2D3, but not 25(OH)D3 or 24,25(OH)2D3, causes an increase in net A-to-B calcium transport across Caco-2 cell monolayers. Moreover, treatment with TFP significantly reduced 1,25(OH)2D3-mediated calcium transport. In contrast, we found that 1,25(OH)2D3 did not have a significant effect on the paracellular transport pathway, as estimated by phenol red movement and TEER. These observations, coupled with our additional observation that calcium transport and calbindin D9k mRNA expression were blocked by actinomycin D treatment, are consistent with a genomic effect of 1,25(OH)2D3 on transcellular A-to-B calcium transport in Caco-2 cells.

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