Vitamin D increases tight-junction conductance and paracellular Ca\(^{2+}\) transport in Caco-2 cell cultures

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Chirayath, Mary V., Leszek Gajdzik, Wolfgang Hulla, Jürg Graf, Heide S. Cross, and Meinrad Peterlik. Vitamin D increases tight-junction conductance and paracellular Ca\(^{2+}\) transport in Caco-2 cell cultures. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G389–G396, 1998.—We investigated the effects of 1α,25-dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] on paracellular intestinal Ca\(^{2+}\) absorption by determination of transepithelial intestinal Ca\(^{2+}\) absorption, as a measure of tight-junction ion permeability and bidirectional transepithelial 45Ca\(^{2+}\) fluxes in confluent Caco-2 cell cultures. The rise of TEER to steady-state levels of –2,000 Ω·cm\(^{-2}\) was significantly attenuated by 1,25(OH)\(_2\)D\(_3\) (by up to 50%) in a dose-dependent fashion between 10\(^{-11}\) and 10\(^{-6}\) M. Synthetic analogs of 1,25(OH)\(_2\)D\(_3\), namely, 1α,25-dihydroxy-16-ene,23-yno-vitamin D\(_3\) and 1α,25-dihydroxy-2,27-hexafluoro-16-ene,23-yno-vitamin D\(_3\), exhibited similar biopotency, whereas their genomics inactive 1-deoxy congeners were only marginally effective. Enhancement of transepithelial conductance of Caco-2 cell monolayers by vitamin D was accompanied by a significant increase in bidirectional transepithelial 45Ca\(^{2+}\) fluxes. Although 1,25(OH)\(_2\)D\(_3\) also induced cellular 45Ca\(^{2+}\) uptake from the apical aspect of Caco-2 cell cultures and upregulated the expression of calbindin-9kDa mRNA, no significant contribution of the Ca\(^{2+}\)-adenosinetriphosphatase-mediated transcellular pathway to transepithelial Ca\(^{2+}\) transport could be detected. Therefore stimulation of Ca\(^{2+}\) fluxes across confluent Caco-2 cells very likely results from a genomic effect of vitamin D sterols on assembly and permeability of tight-junctional complexes.

Intestinal calcium absorption; 1α,25-dihydroxyvitamin D\(_3\); synthetic vitamin D compounds; vitamin D receptor; genomic action; ionic conductance; cellular calcium uptake; calbindin-9kDa; calcium-adenosinetriphosphatase

Experimental Methods

Caco-2 cells. The Caco-2 cell clone AQ, which was used in the present study, was originated in our laboratory by subcloning of an established clone, Caco-2/15 (cf. Ref. 3) after passage 100 by dilution plating. The population doubling time of the Caco-2/AQ clone during the logarithmic growth phase was estimated as 24 h (vs. 36 h of Caco-2/15 clone). The activity of the differentiation marker, alkaline phosphatase, increased during 20 days of confluent growth from an average of 20 to 60 mU/mg cellular protein in Caco-2/AQ cells, whereas the respective values for the parent clone Caco-2/15 were 25 and 190 mU/mg protein.

Cell culture. Caco-2/AQ cells (between passages 20 and 50) were grown either in 24-well Falcon culture plates (Becton-Dickinson, Bedford, MA) or on filters with 0.4-µm pore size (Falcon cell culture inserts), as appropriate, in Dulbecco's modified Eagle's medium [supplemented with 10% fetal calf serum, 4.0 mM glutamine, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 50 U/ml penicillin, and 50 µg/ml streptomycin] at 37°C in a 5% CO\(_2\)-95% air atmosphere.

Vitamin D compounds were dissolved in ethanol and added to cultures so that the final solvent concentration in the medium did not exceed 0.01%.

Measurement of transepithelial electrical resistance. Transepithelial electrical resistance (TEER) of the Caco-2 monolayers was measured by a high-precision technique as described previously in detail (30). Current pulses of 55 µA, 0.5-s duration, were passed across the monolayers with Ag-AgCl electrodes from an ESCOM 486 SX computer equipped with a high-performance Labcard (PCL-818). Resulting voltages were recorded with the aid of a differential amplifier with a high input resistance. Data were corrected for well area (given in Ω·cm\(^{-2}\)).

Transport studies. If not indicated otherwise, the medium used for determination of transepithelial transport of 45Ca\(^{2+}\), 86Rb\(^{+}\), [\(^{14}\)C]mannitol, or [\(^{14}\)C]inulin, respectively, contained (in mM) 134 NaCl, 4.1 KCl, 2.1 CaCl\(_2\), 1.0 KH\(_2\)PO\(_4\), 1.0 MgSO\(_4\), and 12.3 HEPES. pH was adjusted to 7.4 with 1.0 N NaOH. Specific activity of radiotracers was 0.5 µCi/ml.

Transepithelial transport across confluent Caco-2 cells was evaluated as described by Giuliano and Wood (21). Briefly, at time 0, transport buffer containing the radiolabeled solute at an appropriate concentration, i.e., 2.1 mM 45Ca\(^{2+}\), 0.1 mM 86Rb\(^{+}\), 1.0 mM [\(^{14}\)C]mannitol, or 36 µM [\(^{14}\)C]inulin, respectively, was filled into the filter well (1.0 ml) or the outside compartment (3.0 ml) of the filter unit as appropriate. In each case, the concentration of the solute under investigation in the contralateral compartment was zero. The filter plates were shaken horizontally at a frequency of 50 oscillations/min.
at room temperature for 60 min. Linearity of transport rates was monitored by determination of radioactivity in 20-μl aliquots drawn from the contralateral solutions at 15-min intervals.

Uptake studies. Caco-2 cells grown in 24-well plates were allowed to equilibrate with room temperature for 30 min before experimentation. After aspiration of the culture medium, 0.5 ml of a "low-sodium" mannitol buffer containing 45Ca2+ (0.5 μCi/ml) was added into each well. The buffer composition was (in mM) 198 mannitol, 25 KCl, 1.2 NaH2PO4, 25 NaHCO3, 1.2 MgSO4, 0.25 CaCl2, and 20 glucose (24). The uptake experiment was carried out for 10 min under horizontal shaking (50 oscillations/min) at room temperature. For termination of uptake, the transport medium was sucked off, and the cells were washed three times with 1.0 ml of ice-cold phosphate-buffered saline (pH 7.4). Cells were then suspended in 1.0 ml of 1.0 N NaOH and allowed to solubilize by overnight standing at 4°C.

Calbindin-9kDa mRNA isolation and Northern blotting. Total RNA was prepared from cells grown on filters in six-well plates until day 15 according to Ref. 11; 20 μg were used for Northern blotting (as described in Ref. 2). There was no obvious need for the use of semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) as performed by Fleet and Wood (19), since specific message was sufficient for conventional Northern analysis of calbindin-9kDa (CaBP-9kDa) mRNA expression. Probes for human (h) CaBP-9kDa were generated by RT-PCR. The following primers were selected from mRNA expression. Probes for human (h) CaBP-9kDa were generated by RT-PCR. The following primers were selected from hCaBP-9kDa cDNA: Cal9.1.1 (1. for coding), CAACCAGACA CCAGATTAAG; Cal9.2.1 (1. for coding), GTCTCTGAG GAAGCTGAAGA; Cal9.3.0 (0. for reverse), GACCAGACACT CCAAGTCAAT; Cal9.4.0 (0. for reverse), TAAGCTGAA ACCAGACCAG. The primes’ specificity was determined by searching data bases with the respective sequences (FASTA), and primers with 100% homology to only hCaBP-9kDa were accepted. Products of ~380 and 290 bp with primers Cal9.1. and Cal9.4. ("outside") and Cal9.2. and Cal9.3. ("inside", respectively) were obtained after 30 cycles. The longer PCR product was then reamplified with the inside primers. The expected length together with a characteristic restriction fragment pattern definitely identified the product as hCaBP-9kDa (RT-PCR from human brain cDNA yielded no product and thus served as a negative control). The PCR product was cloned into a pCRII vector (Invitrogen) and cDNA yielded no product and thus served as a negative control.

RESULTS

Development of paracellular permeability in confluent Caco-2 cells. Mucosal-to-serosal transepithelial transport of extracellular markers, namely, [14C]inulin (~5,000 mol wt) and [14C]mannitol (182.2 mol wt), was determined in parallel to 45Ca2+ transfer across confluent Caco-2 cell layers. As shown in Fig. 1, exposure of Caco-2 cells to 10-8 M 1,25(OH)2D3 for 2 wk post confluence had a distinct effect on the extent to which extracellular markers of different molecular weight could penetrate the Caco-2 cell layer. Although exposure to the steroid hormone had no effect whatsoever on transfer of the high molecular weight compound, inulin, a small but significant vitamin D-related increment of transport of the considerably smaller molecule mannitol could be observed. Expectedly, 1,25(OH)2D3 elicited an approximately threefold rise in transepithelial transport of 45Ca2+ (Fig. 1).

This observation suggested to us that 1,25(OH)2D3 could attenuate the closure of intercellular junctions during postconfluent differentiation to such an extent that the passage of bulky extracellular markers such as inulin would be severely restricted, whereas smaller molecules and ions such as mannitol, or even more importantly Ca2+, could still traverse the cell layer on a paracellular route.

To substantiate this notion, we measured TEER of confluent Caco-2 cell layers as a highly sensitive parameter of paracellular permeability simultaneously with mucosal-to-serosal transepithelial transport of [14C]mannitol and 45Ca2+ at different time points after confluence.

Figure 2A shows that 1,25(OH)2D3 markedly retards the development of TEER of Caco-2 cell layers during postconfluent growth. A significant effect of the sterol becomes visible at day 4. Figure 2B shows that the rate

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**Fig. 1.** Time course of [14C]inulin (○, ■), [14C]mannitol (○, △), and 45Ca2+ (□, ■) transfer across confluent Caco-2 cells and effect of 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3]. Cells were grown until day 14 post confluence. Solid symbols, presence of 10-8 M 1,25(OH)2D3 in culture medium from day 1 after confluence on; open symbols, untreated controls. Concentrations in transport buffer (apical compartment only): inulin, 36 μM; mannitol, 1.0 mM; Ca2+, 2.1 mM. Data from a typical experiment are shown as means from 6 determinations ± SE (vertical bars).
of \([^{14}C]\)mannitol is inversely related to the development of TEER (32). Consequently, treatment with \(1,25(\text{OH})_2\text{D}_3\) made Caco-2 cells to a small but significant extent \((P < 0.05)\) more permeable to \([^{14}C]\)mannitol (Fig. 2B). A similar relation to TEER was observed in the case of transepithelial \(45\text{Ca}^{2+}\) transport (Fig. 2C). The ~50% reduction of TEER observed in \(1,25(\text{OH})_2\text{D}_3\) treated Caco-2 cultures between days 10 and 15 past confluence (Fig. 2A) was accompanied by an about threefold increase in the amount of mucosal-to-serosal \(\text{Ca}^{2+}\) transfer (Fig. 2C).

A dose-response study was carried out at two different time points after Caco-2 cells reached confluence (Fig. 3). On day 4, a significant reduction of TEER and a concomitant rise in \(\text{Ca}^{2+}\) transport was observed only in cells exposed to the highest \(1,25(\text{OH})_2\text{D}_3\) concentration tested, i.e., \(10^{-8}\) M, whereas, on day 12, linear dose responses were observed at sterol concentrations between \(10^{-10}\) and \(10^{-9}\) M.

A plot of transport rate vs. conductance (i.e., \(1/\text{resistance}\)) generated from single measurement values at various time points after confluence (Fig. 4), yielded identical curves for both \(1,25(\text{OH})_2\text{D}_3\)-treated and untreated Caco-2 cell layers. The absence of any vitamin D-related transport increment at a given conductance value strongly indicates that \(1,25(\text{OH})_2\text{D}_3\) had no visible influence on apical-to-basolateral \(\text{Ca}^{2+}\) transfer other than on the paracellular route.

If transepithelial \(\text{Ca}^{2+}\) transport as measured is solely determined by the capacity of the paracellular route, then \(\text{Ca}^{2+}\) transfer both in the apical-to-basolateral and in the opposite direction should occur to an equal extent. In some experiments, we measured also the influence of \(1,25(\text{OH})_2\text{D}_3\) on basolateral-to-apical transepithelial \(\text{Ca}^{2+}\) transport. At any time point, transport rates across either \(1,25(\text{OH})_2\text{D}_3\)-treated or untreated Caco-2 cell layers were equal to respective flux rates in the opposite direction (cf. also Table 1). Again, an identical relationship between \(\text{Ca}^{2+}\) transport rates and conductance as measure of paracellular permeability was observed in both hormone-treated and control groups (Fig. 4).

Effect of \(1,25(\text{OH})_2\text{D}_3\) analogs on TEER of confluent Caco-2 cells. Caco-2 cells respond not only to \(1,25(\text{OH})_2\text{D}_3\) but also to a number of its synthetic analogs by changes in growth and morphological appearance, provided that those compounds bear a \(1\alpha\)-hydroxy group (5), which is a prerequisite for binding to the vitamin D receptor (VDR) and hence for genomic activity. We therefore tested the effect of \(1,25(\text{OH})_2\text{D}_3\)
and of two potent antimitogenic compounds, 1,25(OH)2-16ene,23yne-D3, and 1,25(OH)2-26,27-F6-16ene,23yne-D3, as well as of the respective 1α-deoxy compounds, [25(OH)D3, 25-hydroxyvitamin D 3], and 25(OH)-16ene,23yne-D3, and 25(OH)-26,27-hexafluoro-16-ene,23-yne-vitamin D3, from confluence on. Data are means ± SE (n = 6) from a typical experiment. If not labeled NS (not significant), treatment groups were statistically different from control group at least at P < 0.05. 25(OH)D3, 25-hydroxyvitamin D3; 25(OH)-16ene,23yne-D3, 25-hydroxy-16-ene,23-yne-vitamin D3; 25(OH)-26,27-F6-16ene,23yne-D3, 25-hydroxy-26,27-hexafluoro-16-ene,23-yne-vitamin D3; 1,25(OH)2-16ene,23yne-D3, 1α,25-dihydroxy-16-ene,23-yne-vitamin D3; 1,25(OH)2-26,27-F6-16ene,23yne-D3, 1α,25-dihydroxy-26,27-hexafluoro-16-ene,23-yne-vitamin D3.

Table 1. Short-term effect of 1,25(OH)2D3 on TEER and bidirectional 45Ca2+ transport across confluent Caco-2 cell layers

<table>
<thead>
<tr>
<th>Duration of Treatment, h</th>
<th>125(OH)2D3 (10-8 M)</th>
<th>TEER (Ω·cm²)</th>
<th>Apical to basolateral</th>
<th>Basolateral to apical</th>
<th>Transcellular Ca2+ Transport, nmol·h⁻¹·well⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>1.432 ± 0.50</td>
<td>11.2 ± 0.04</td>
<td>14.3 ± 1.11</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>–</td>
<td>1.386 ± 0.14</td>
<td>9.2 ± 0.04</td>
<td>9.0 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>+</td>
<td>1.436 ± 0.30</td>
<td>10.3 ± 0.33</td>
<td>9.9 ± 0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.407 ± 0.10</td>
<td>9.8 ± 0.60</td>
<td>9.3 ± 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.886 ± 0.10</td>
<td>16.0 ± 0.60</td>
<td>15.5 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>+</td>
<td>1.466 ± 0.80</td>
<td>8.9 ± 0.44</td>
<td>11.2 ± 0.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.075 ± 0.32</td>
<td>16.6 ± 1.72</td>
<td>17.9 ± 0.40</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6) from a typical experiment. 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; TEER, transepithelial resistance.

development of TEER of postconfluent Caco-2 cell layers. Although 25(OH)D3, with the exception of day 5, had no significant effect, 25(OH)-16ene,23yne-D3, and 25(OH)-26,27-F6-16ene,23yne-D3, from day 8 or 5 on, respectively, significantly reduced TEER by an average of ≤10%. In contrast, 1,25(OH)2D3 and both synthetic 1α-hydroxylated analogs reduced TEER to ≤50% of control levels at any time point. It should be noted that the decrement in TEER induced by the synthetic 1α-hydroxyvitamin D compounds tended to be even higher than that induced by 1,25(OH)2D3.

Time course of 1,25(OH)2D3 effects on TEER and transcellular Ca2+ transport. The increase in TEER during growth of confluent Caco-2 cells in all likelihood reflects the development of tight junctions (cf. Ref. 28). It was therefore of interest to know whether vitamin D would affect not only the assembly of intercellular junctions during confluent cell growth but also influence their barrier function in a more advanced state of development. Thus, in another series of experiments, Caco-2 cells were allowed to grow for 12 days past confluence before treatment with 1,25(OH)2D3 was begun. Table 1 shows that, after 48–72 h, a highly significant reduction of TEER with a concomitant rise in transepithelial Ca2+ transport could be observed. It should be noted that Ca2+ transport in the mucosal-to-serosal as well as in the opposite direction was influenced by the hormone to the same extent (Table 1).

Effect of 1,25(OH)2D3 on cellular 45Ca2+ uptake and CaBP-9kDa mRNA expression. To evaluate a possible contribution of the transcellular route to vitamin D-related transepithelial Ca2+ transport as measured, we
determined the effect of 1,25(OH)_{2}D_{3} and analogs on Ca^{2+} uptake by Caco-2 cells at different growth stages (Fig. 6). Basal cellular Ca^{2+} uptake in vitamin D-free control cultures conspicuously increased during transition from the log growth phase into the confluent state. During this time period, the 1α,25-dihydroxyvitamin D compounds under investigation were most effective in raising cellular 45Ca^{2+} accumulation, whereas the 1α-deoxy compound, 25(OH)-16ene,23yne-D_{3}, had no effect at all.

In another experiment, the effect of 1,25(OH)_{2}D_{3} on cellular Ca^{2+} accumulation was studied in confluent filter-grown Caco-2 cells, which were selectively exposed to the radiotracer 45Ca^{2+} either on their apical (i.e., mucosal) or basolateral (i.e., serosal) aspect, respectively. As expected, 1,25(OH)_{2}D_{3} when added on day 12 past confluence at 10^{-8} M, after 72 h raised cellular 45Ca^{2+} accumulation due to uptake from the mucosal compartment from 6.8 ± 0.04 to 15.2 ± 0.3 nmol·h^{-1}·well^{-1} (n = 6, P < 0.01) but had no significant effect on uptake from the contralateral compartment, which was raised from 18.0 ± 1.8 to only 21.4 ± 0.3 nmol·h^{-1}·well^{-1} (n = 6, P > 0.05).

In the same experiment, we determined CaBP-9kDa mRNA levels by Northern blot analysis (Fig. 7). Caco-2 cells were able to express CaBP-9kDa message even in the absence of 1,25(OH)_{2}D_{3}. As expected (cf. Refs. 18, 19), a two- to threefold rise in mRNA levels was induced by 10^{-8} to 10^{-8} M 1,25(OH)_{2}D_{3} within 48–72 h.

Ca^{2+}-adenosinetriphosphatase inhibition and transepithelial Ca^{2+} transport. Because the last step of mucosal-to-serosal transcellular Ca^{2+} transport involves extrusion of Ca^{2+} across the basolateral aspect of the cell by the Ca^{2+}-adenosinetriphosphatase (ATPase), we sought to evaluate the contribution of active Ca^{2+} pumping to net transepithelial transport by blocking the activity with a potent inhibitor, calmidazolium. The data collated in Table 2 show that, apart from the fact that pretreatment with the inhibitor had no influence on TEER in either controls or 1,25(OH)_{2}D_{3}-treated Caco-2 cells, a block of the Ca^{2+} pump did not change the extent of basal mucosal-to-serosal transepithelial Ca^{2+} transfer as measured but, even more important, by no means reduced its 1,25(OH)_{2}D_{3}-related increment.

1,25(OH)_{2}D_{3} and transepithelial 86Rb\textsuperscript{+} transport. Further proof for the assumption that 1,25(OH)_{2}D_{3} can modulate ion transport on the paracellular route was obtained when we measured transepithelial 86Rb\textsuperscript{+} transport across confluent Caco-2 cell layers (Table 3). 86Rb\textsuperscript{+} is widely used as a substitute for K\textsuperscript{+} for assessment of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in whole cell preparations. It should be noted that, under the experimental conditions employed, transepithelial 86Rb\textsuperscript{+} transport was completely insensitive to ouabain treatment (Table 3). Because this excludes any contribution from the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase-mediated transcellular pathway, 86Rb\textsuperscript{+} transport as measured mainly reflects ion flux on a paracellular route. The data collated in Table 3 therefore strongly suggest that stimulation of 86Rb\textsuperscript{+} transport across confluent Caco-2 cell layers in the serosal-to-mucosal direction by the steroid hormone occurs in parallel with reduction of TEER during postconfluent cell growth.

Table 2. Ca^{2+}-ATPase inhibition and transepithelial 45Ca^{2+} transport in Caco-2 cells

<table>
<thead>
<tr>
<th>Addition to Culture</th>
<th>Calmidazolium Pretreatment</th>
<th>TEER, Ω·cm\textsuperscript{-2}</th>
<th>Transepithelial 45Ca\textsuperscript{2+} Transport, nmol·h\textsuperscript{-1}·well\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>−</td>
<td>1,949 ± 61</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td>1,25(OH)<em>{2}D</em>{3}</td>
<td>+</td>
<td>1,935 ± 32</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>(10^{-8} M)</td>
<td></td>
<td>1,024 ± 19</td>
<td>28.3 ± 0.2</td>
</tr>
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</table>

Data are means ± SE (n = 6) from a typical experiment. Pretreatment with 100 µM calmidazolium was for 30 min before initiation of 45Ca\textsuperscript{2+} transport. Inhibitor was included at the same concentration that was also in outer, i.e., basolateral compartment of filter unit (see EXPERIMENTAL METHODS).
Table 3. Effect of 1,25(OH)2D3 on paracellular 
Rb flux and TEER in confluent Caco-2 cells

<table>
<thead>
<tr>
<th>Day Past Confluence</th>
<th>1,25(OH)2D3 in Culture</th>
<th>Medium Ouabain</th>
<th>TEER, Ω·cm²</th>
<th>Serosal-to-Mucosal Rb Transport, pmol·min⁻¹·well⁻¹</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>1.497 ± 0.25</td>
<td>37.3 ± 0.6</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.504 ± 0.37</td>
<td>37.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.063 ± 0.21</td>
<td>49.2 ± 1.2</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>1.058 ± 0.25</td>
<td>52.0 ± 0.6</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.609 ± 0.15</td>
<td>39.3 ± 1.0</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.861 ± 0.55</td>
<td>37.8 ± 1.7</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>798 ± 58</td>
<td>59.6 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>794 ± 50</td>
<td>59.9 ± 1.2</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>2.121 ± 0.25</td>
<td>25.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>2.054 ± 0.42</td>
<td>20.8 ± 0.9</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.163 ± 0.25</td>
<td>41.4 ± 1.7</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.092 ± 0.29</td>
<td>42.5 ± 1.5</td>
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</table>

Data are means ± SE (n = 6) from a typical experiment. 1,25(OH)2D3 concentration in culture medium was 10⁻⁸ M. Preincubation with 1.0 mM ouabain was for 4 h before initiation of Rb⁻⁻⁻ transport. Inhibitor was included at same concentration also in transport buffer.

DISCUSSION

Caco-2 cells, though originally derived from a human colon adenocarcinoma, are still able to undergo spontaneous differentiation into enterocyte-like cells. Thereby Caco-2 cells form confluent monolayers consisting of well-polarized cells with tight junctions and a typical apical brush border (28).

It is generally accepted that transepithelial electric conductance across Caco-2 cell layers is mainly determined by the ionic permeability of the intercellular junctions which develop during postconfluent cell growth (28). The fact that the paracellular route is the predominant pathway of transepithelial ion flux can be inferred from the observations that ~80% of total electrical resistance of Caco-2 cells is located in the mucosal membrane and that, in addition, its Na⁺ conductance is very limited (22). Changes in TEER can also not be explained by activation of Na⁺-dependent α-glucose cotransport, because the Caco-2 cell line used in the present study is devoid of any Na⁺-dependent α-glucose transport activity (10, 22), and in addition, no α-glucose was present in the incubation medium. Thus the decline in TEER largely reflects an effect on tight junction-mediated paracellular ion permeability (27). The present study documents that 1,25(OH)₂D₃ as well as its synthetic D-ring and side-chain-modified analogs substantially reduce TEER of confluent Caco-2 cells. This must be considered as clear evidence for the ability of genomically active vitamin D compounds to increase bidirectional paracellular flux of all ion species including Ca²⁺. In this respect, it is interesting to note that Favus et al. (16) had observed that 1,25(OH)₂D₃ caused a significant increase of tissue conductance and, most notably, also of bidirectional mannitol fluxes in the duodenum and descending colon of rats, whereas Cross et al. (12) reported on stimulation of paracellular ion transport, i.e., Na⁺, K⁺, and Rb⁺, in organ-cultured embryonic chick small intestine.

Caco-2 cells express VDR mRNA and protein during the log growth phase as well as after confluence (20, 23). This is apparently the basis for the action on paracellular ion permeability of vitamin D compounds, since all 1α-deoxvitan D compounds under investigation were either completely ineffective in reducing TEER, namely, 25(OH)-D₃, or, like the two synthetic compounds, 25(OH)-16ene,23yne-D₃ and 25(OH)-26,27-F₆-16ene,23yne-D₃, showed only marginal activity compared with their 1α-hydroxylated congeners (cf. Fig. 5). Because the 1α-hydroxy group mediates high-affinity binding to the VDR (cf. Ref. 6), it is reasonable to assume that the observed effects on TEER of 1,25(OH)₂D₃ and its two synthetic side-chain- and D-ring-modified analogs result from a genomic rather than from a nongenomic action. The latter possibility seems unlikely also for a number of other reasons. First, typical nongenomic effects of 1,25(OH)₂D₃ (for review see, e.g., Ref. 7) involve interactions with plasma membrane activities and are observed within seconds or minutes, whereas reduction of TEER requires at least 48-h exposure to the hormone (cf. Table 1). Second, a rapid membrane action of 1,25(OH)₂D₃ cannot be easily reconciled with the observation that the sensitivity of Caco-2 cells varies with ongoing differentiation between days 4 and 12 past confluence (cf. Fig. 3). It has been shown, however, that the expression of genomic effects of 1,25(OH)₂D₃, particularly in enterocytes, can depend to a large extent on the degree of their differentiation (13). Third, 25(OH)-16ene,23yne-D₃ was shown to be most potent in eliciting nongenomic effects such as activation of voltage-gated Ca²⁺ channels in rat osteosarcoma cells (15), whereas the same analog was only weakly effective in attenuating TEER (cf. Fig. 5). Fourth, it is conceivable that the observed small effects of synthetic 1-deoxvitan D compounds on TEER of Caco-2 cells reflect their small genomic potency due to the ability to bind weakly to the VDR (6, 15) or, fifth, result from conversion into genomically active 1-hydroxy compounds. Although substantial 25-hydroxyvitamin D₃-1-hydroxylase activity has been observed only in serum-free cultures of Caco-2 cells (14), it is conceivable that, even under the culture conditions employed in the present study, a small fraction of the 25-hydroxy compounds tested is converted to respective 1α-hydroxy derivatives, which could then be responsible for the observed effects on TEER (Fig. 5).

Both assembly and barrier properties of tight junctions depend on the formation of a bipartite functional complex with adjacent adherens junctions as well as on an appropriate organization of the latter with the actin cytoskeleton (1, 26). Fialka et al. (17) showed that estrogen-related upregulation of the c-jun protooncoprotein diminishes TEER in mammary epithelial cells and, at the same time, disrupts the polarized expression of the tight junction-associated protein zonin-1 as well as of the constituents of adherens junctions, E-cadherin and β-catenin. Because the c-jun protooncopogene is also a well-known target for signaling from the VDR (9, 25), we suggest that upregulation of c-jun expression could also explain the observed effects of genomically active vitamin D compounds on tight-junctional permeability.
of Caco-2 cells. In fact, we have obtained evidence from Western blot analysis that treatment with $10^{-8}$ M 1,25(OH)$_2$D$_3$ for 5 days leads to reduced expression of E-cadherin in Caco-2 cells (unpublished results).

A strong argument for the notion that vitamin D stimulates transepithelial Ca$^{2+}$ transport by an increase in junctional ion permeability rather than by stimulation of transcellular calbindin-mediated transport, as suggested by Fleet et al. (18, 19), can be derived from the following observations: 1) an identical relationship between TEER or conductance, respectively, and Ca$^{2+}$ transport exists in untreated and vitamin D-treated Caco-2 cell cultures, and hence no conductance-independent vitamin D-related increment exists; 2) vitamin D has an identical effect on apical-to-basolateral as well as on basolateral-to-apical Ca$^{2+}$ fluxes, which would not be the case if there were a major contribution from vectorial transcellular calbindin-mediated transport that proceeds exclusively in the apical-to-basolateral direction; and 3) the effect of vitamin D is not specific for Ca$^{2+}$ transport but is visible also on bidirectional Rb$^+$ fluxes, which are certainly not calbindin mediated.

As far as the existence of a major transcellular Ca$^{2+}$ path in confluent Caco-2 cells is concerned, we were able to confirm the observation of Surendran et al. (29) that blocking Ca$^{2+}$ extrusion across the basolateral membrane by Ca$^{2+}$-ATPase inhibition does not alter the extent of transepithelial Ca$^{2+}$ transport. Because this is valid also for 1,25(OH)$_2$D$_3$-treated Caco-2 cells (cf. Table 2), this observation must be considered as additional support for the assumption that vitamin D affects Ca$^{2+}$ transport mainly through its effect on tight-junctional ion permeability.

In probing the vitamin D sensitivity of the consecutive steps of apical-to-basolateral transcellular Ca$^{2+}$ transport, we could show that cellular Ca$^{2+}$ uptake from the apical aspect of confluent Caco-2 cell layers involves a genomic action of vitamin D sterols. Furthermore, consistent with the results of Fleet et al. (18, 19), 1,25(OH)$_2$D$_3$ upregulates calbindin-9kDa mRNA levels (Fig. 7). However, it must be borne in mind that as long as direct measurement of human calbindin-9kDa protein in Caco-2 cells is not available, it remains questionable whether the vitamin D actions on mucosal Ca$^{2+}$ influx and CaBP-9kDa mRNA are of a magnitude to efficiently raise the rate of transcellular transport of Ca$^{2+}$.

Another explanation for the difference in the interpretation of our results and those of Fleet et al. (18, 19) lies in the fact that these authors did not observe any effect of vitamin D sterols on transepithelial transfer of phenol red, which they used as a marker for paracellular permeability. However, the relatively high molecular weight and negative charge of this compound may have compromised its use to detect changes in paracellular permeability of ions with a much smaller atomic radius, such as Ca$^{2+}$. Because of the lack of any substantial contribution of the transcellular route to transepithelial Ca$^{2+}$ transport, the Caco-2 system could serve as an excellent model for the study of vitamin D effects on intestinal Ca$^{2+}$ absorption via the paracellular route (for review, see Ref. 31).

The authors thank Teresa Manhardt for skillful technical assistance.

These investigations were supported by Grant P-10133-MED from the Austrian Science Foundation.

M. V. Chirayath was on leave of absence from Dept. of Physiology, PSG Medical College, Coimbatore, South India, and was the recipient of a fellowship of the International Academy of Pathology, Austrian Section.

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Received 12 December 1996; accepted in final form 20 October 1997.

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