Osteoblast Ca\(^{2+}\) permeability and voltage-sensitive Ca\(^{2+}\) channel expression is temporally regulated by 1,25-dihydroxyvitamin D\(_3\)

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VSCCs are present in all excitable tissues and at lower levels in most nonexcitable cell types, in which they mediate the influx of Ca\(^{2+}\) in response to membrane depolarization and regulate intracellular functions, including excitation-secretion, gene transcription, neurotransmitter release, and cell differentiation. These responses are fine-tuned to a specific temporal pattern of Ca\(^{2+}\) entry (17, 20) that is accomplished by expression of unique subtypes of VSCCs, each of which differs regarding the kinetics of activation and inactivation, pharmacology, and tissue distribution. In osteoblasts, L-type VSCCs consist of four discrete protein subunits (\(\alpha_1, \beta_2, \delta\), and \(\beta\)) (4). The \(\alpha_1\)-subunit provides the pore through which Ca\(^{2+}\) ions enter the cell and can generate a Ca\(^{2+}\) current in the absence of the other subunits (35). At present, at least 10 distinct \(\alpha_1\)-subunits have been identified (37). Physiological and pharmacological studies have demonstrated functional similarities among various \(\alpha_1\)-subunits that allow VSCCs to be classified into high-voltage activated (L-, P/Q-, N, and R-type) and low-voltage activated (T-type) types (39). L-type Ca\(^{2+}\) currents are mediated by VSCCs containing Cav1.1 \(\alpha_1\)-subunit, Cav1.2 \(\alpha_1\)-subunit, Cav1.3 \(\alpha_1\)-subunit, and Cav1.4 \(\alpha_1\)-subunits, while Cav2.1 \(\alpha_1\), Cav2.2 \(\alpha_1\), and Cav2.3 \(\alpha_1\) (P/Q-, N-, and R-type VSCCs, respectively) compose the remainder of the high-voltage activated VSCC family. The low-voltage activated T-type VSCCs include Cav3.1 \(\alpha_1\), Cav3.2 \(\alpha_1\), and Cav3.3 \(\alpha_1\). Low- and high-voltage activated channels share <25% sequence similarity at the protein level and display remarkably different functional properties.

Previous studies conducted at our laboratory have definitively established that the L-type VSCC Cav1.2 \(\alpha_1\)-subunit is the primary site for Ca\(^{2+}\) influx into the proliferating osteoblast (9, 27) and also have shown that application of 1,25(OH)\(_2\)D\(_3\) increases plasma membrane permeability to Ca\(^{2+}\) within milliseconds by shifting the threshold of activation toward the resting potential and increasing the mean open time of the L-type VSCC (9, 25). Interestingly, rat osteoblastic cells (ROS17/2.8) treated for 24–48 h with 1,25(OH)\(_2\)D\(_3\) reduced L-type VSCC transcription levels while they increased expression of differentiation markers, including osteopontin and osteocalcin (30). Prior observations also have shown that terminally differentiated osteocytes express Cav3.2 \(\alpha_1\), but not Cav1.2 \(\alpha_1\) (41), although the effects of 1,25(OH)\(_2\)D\(_3\) on the expression of this channel have not been examined.

In this study, we used quantitative real-time PCR (QPCR) and specific immunostaining approaches to identify the spectrum of VSCC \(\alpha_1\)-subunits expressed in murine MC3T3-E1 osteoblasts (25, 30). Prior observations also have shown that terminally differentiated osteocytes express Cav3.2 \(\alpha_1\), but not Cav1.2 \(\alpha_1\) (41), although the effects of 1,25(OH)\(_2\)D\(_3\) on the expression of this channel have not been examined.

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osteoblastic cells. In addition, we examined the effects of 1,25(OH)\(_2\)D\(_3\) on VSCC \(\alpha\)_1-subunit expression. Finally, \(^{45}\)Ca\(^{2+}\) influx assays were performed in the presence of various pharmacological inhibitors specific for VSCC classes to determine how treatment with 1,25(OH)\(_2\)D\(_3\) altered the properties of Ca\(^{2+}\) permeability.

**MATERIALS AND METHODS**

Cell culture. MC3T3-E1 cells, a gift from Dr. Renny T. Francesch (Department of Periodontics/Prevention/Geriatrics, University of Michigan School of Dentistry, Ann Arbor, MI), were plated at 5,000 cells/cm\(^2\) and maintained in \(\alpha\)-MEM containing ribonucleosides and deoxyribonucleosides supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10 mM HEPES buffer. All culture reagents were purchased from Invitrogen (Carlsbad, CA). Cultures were maintained in a 37°C humidified atmosphere with 5% CO\(_2\). The medium was changed every 3 days, and the cell lines were passaged at 80% confluence using trypsin-EDTA.

For 1,25(OH)\(_2\)D\(_3\) treatment, cells were plated into T-75 culture flasks (Corning, Corning, NY) and cultured for 72 h in serum-containing medium. The cells were then rinsed twice with PBS and fresh serum-free medium was added that contained either 1,25(OH)\(_2\)D\(_3\) or an equal amount of vehicle 0.01% ethanol (vol/vol).

**RNA isolation and RT-PCR.** Total RNA was extracted from MC3T3-E1 cell cultures at 80% confluence using the RNAeasy kit, which we obtained from Qiagen (Valencia, CA), according to the manufacturer’s instructions. First Choice total RNA from normal mouse tissue was purchased from Ambion (Austin, TX) and contained RNA from mouse liver, brain, thymus, heart, lung, spleen, testis, ovary, kidney, and embryonic day 10 tissue. Total RNA was reverse transcribed using the Advantage for RT-PCR kit available from BD Biosciences Clontech (Palo Alto, CA). Total RNA (1 \(\mu\)g) was reverse transcribed for 45 min at 40°C. The enzyme then was heat inactivated at 95°C for 15 min.

QPCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer’s instructions, except that fluorescein reference dye (1 nM) was added to each reaction to normalize the instrument’s optics and to compensate for variations in fluorescence among wells. The primer sequences used, which are listed in Table 1, were designed on the basis of published species-specific sequences. Standards were generated by cloning the PCR product into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen). Isolated plasmid was linearized using EcoRV, quantitated, and diluted for use as standards in QPCR. QPCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA), and true mRNA levels were calculated exactly as recommended by the manufacturer. Data were analyzed using Prism 3.0 software (GraphPad, San Diego, CA). For confirmation of specificity, nonlinear RT-PCR also was performed with the same primer sets. After 10 min incubation at 95°C, the cycling conditions were as follows: denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension for 60 s at 72°C for 45 cycles, well beyond the linear range for most channel subunits. All PCR products were sequence verified using the BioResource Center at Cornell University (available online at http://www.brc.cornell.edu/brcinfo/index.php).

**Immunodetection of \(\alpha\)_1-subunit expression using confocal microscopy.** Detection of \(\alpha\)_1-subunits was performed using affinity-purified rabbit PAbs directed against the various \(\alpha\)_1-subunits. Primary antibodies and blocking peptides specific for Cav2.1 (\(\alpha\)\(_{1A}\)), Cav2.2 (\(\alpha\)\(_{1B}\)), Cav2.3 (\(\alpha\)\(_{1C}\)), Cav3.1 (\(\alpha\)\(_{1D}\)), Cav3.2 (\(\alpha\)\(_{1E}\)), Cav3.3 (\(\alpha\)\(_{1F}\)), and Cav3.4 (\(\alpha\)\(_{1G}\)) were purchased from Alomone Labs (Jerusalem, Israel). Western blots showed no cross-reactivity of these antibodies.

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**Table 1. Oligonucleotide sequences used for QPCR**

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<th>mRNA Species</th>
<th>Sequence</th>
<th>5’ Position</th>
<th>GenBank Accession No.</th>
<th>PCR Product, bp</th>
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<td>Cav2.1 ((\alpha)(_{1A}))</td>
<td>Forward 5’- ATCCCTATGTTGAAGGTTCC-3’</td>
<td>1,807</td>
<td>AB025352</td>
<td>105</td>
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<td>Reverse 5’- GCAGAAGTCCTGGGCTA-3’</td>
<td>1,911</td>
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<td>Forward 5’- TGCAGGCATGTTGAAGGTTCC-3’</td>
<td>821</td>
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<td>Reverse 5’- ACAGGAGAAGTCCTGGGCTA-3’</td>
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<td>Reverse 5’- GCGAGCAGCTGTTGAGGAAAGGTTCC-3’</td>
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QPCR, quantitative real-time PCR; Cav, cardiac subtype of L-type voltage-sensitive Ca\(^{2+}\) channel.
Cav1.2 (α1C), Cav1.3 (α1D), Cav2.3 (α1E), and Cav1.4 (α1F) were purchased from Alomone Laboratories (Jerusalem, Israel). The rabbit anti-mouse PAb to Cav3.2 (α1M) was designed as part of this work and was generated against the peptide sequence [C]HLEEDFDKLRD-VRATE located in the intracellular loop between transmembrane domains II and III. The affinity-purified antibody was obtained from a commercially available source (Invitrogen Life Technologies).

MC3T3-E1 (5,000 cells/well) were plated onto eight-well Lab-Tek chamber slides (Nalg Nunc International, Naperville, IL). The cells were grown overnight in a 37°C incubator containing 5% CO2. After 24 h, the cells were washed three times with PBS and then fixed in 4% (vol/vol) methanol-free paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1% (vol/vol) PBS for 30 min on ice. After fixation, the cells were rinsed with 0.01% (wt/vol) sodium azide in PBS for 5 min on ice. To permeabilize and block nonspecific binding, the cells were incubated in blocking buffer containing 5% (vol/vol) normal donkey serum, 0.3% (vol/vol) Triton X-100, and PBS for 30 min at room temperature. The primary antibody was diluted 1:50 with 1% (vol/vol) normal donkey serum, 0.1% (wt/vol) BSA, and 0.01% (wt/vol) sodium azide in PBS and then applied to the fixed cells and incubated for 1 h at room temperature. After being washed three times for 10 min each with 1% (vol/vol) normal donkey serum and PBS, the cells were incubated in the dark for 1 h at room temperature using FITC-conjugated donkey anti-rabbit IgG (1:100 dilution; Jackson ImmunoResearch, West Grove, PA). The cells were counterstained for 10 min in the dark with the nuclear dye ToPro3 (Molecular Probes, Eugene, OR) diluted 1:4,000 in PBS. The cells were washed three times for 5 min each in PBS and stored at 4°C until being studied. The fluorescence was analyzed using an inverted microscope linked to a confocal scanning unit (LSM 510; Carl Zeiss, Oberkochen, Germany). To determine the specificity of staining, images were compared with cells that had been incubated with FITC-conjugated donkey anti-rabbit IgG in the absence of primary antibody. Immunostained cells also were compared using slides stained with primary antibody that had been preincubated for 1 h with 1 μg of antigenic peptide per 1 μg of primary antibody (peptide block).

Detection of α1-subunit mRNA in osteoblastic cells. To identify the VSCC α1-subunits expressed in osteoblastic cells, QPCR was performed on RNA isolated from 80% confluent growth-phase MC3T3-E1 cells. The primer sequences used are listed in Table 1 and were chosen on the basis of sequence information available in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Sequence analysis of PCR amplimers revealed that each shared at least 98% sequence identity with the published sequence for that subunit. The amplification products from each α1-subunit primer produced from MC3T3-E1 cells are shown in Fig. IA. Note that these amplification products are based not on quantitative gels but on extended cycles (45) optimized to produce a roughly identical amplimer product for sequence analysis. As shown, growth-phase MC3T3-E1 cells contained at least minimal levels of transcripts encoding all of the known α1-subunits except for Cav1.4 (α1F), which to date has been found only in RNA isolated from retinal cells. To ensure that each primer set was capable of generating a specific product, RT-PCR also was performed using an RNA mixture containing RNA from mouse liver, brain, thymus, heart, lung, spleen, testis, ovary, kidney, and embryonic day 10 tissue (Fig. IB). Each primer set generated product bands identical in size to the bands detected in MC3T3-E1 cells, and sequence analysis confirmed that...
positive control RNA generated PCR products that were the same as those from MC3T3-E1 cells. RT-PCR analysis performed on the positive control RNA confirmed that the primer set specific for the Cav1.4 α1D-subunit produced a 121-bp product that had the proper sequence, confirming that MC3T3-E1 cells do not produce transcripts encoding the Cav1.4 α1D-subunit.

Effects of 24-h exposure of 1,25(OH)2D3 on VSCC α1-subunit mRNA expression. QPCR was performed to quantify mRNA levels encoding various α1-subunits after 24-h treatment with 1,25(OH)2D3 or vehicle (0.01% ethanol). After 24-h incubation in serum-free medium, 80% confluent MC3T3-E1 cell cultures were treated with 0.1, 1, 10, or 100 nM 1,25(OH)2D3 or vehicle (0.01% ethanol), or they were left untreated. After 24 h, total RNA was isolated and QPCR was performed. To detect changes in expression levels, the QPCR results for the VSCCs expressed in MC3T3-E1 cells were normalized to untreated control values for each primer set. The relative L-type VSCC Cav1.2 α1C-subunit transcript levels are shown in Fig. 2. There was no difference in Cav1.2 α1C-subunit transcript levels in MC3T3-E1 cells treated for 24 h with 0.1 nM 1,25(OH)2D3, cells treated with vehicle (ethanol), or cells that were left untreated. When treated for 24 h with 1, 10, or 100 nM 1,25(OH)2D3, Cav1.2 α1C-subunit transcription levels were reduced to approximately one-half the levels found in untreated MC3T3-E1 cells, all of which were statistically significant. Cav1.3 α1P-subunit transcription levels in MC3T3-E1 cells treated with 0.1, 1, 10, or 100 nM 1,25(OH)2D3 did not differ from transcription levels in vehicle-treated and untreated cells (Fig. 2). Similarly, the other L-type VSCC present in osteoblastic cells, the Cav1.1 α1S-subunit, also was unaffected by 24-h 1,25(OH)2D3 treatment (Fig. 2). These data suggest that the Cav1.2 α1C-subunit is the only L-type VSCC present in MC3T3-E1 osteoblastic cells, the expression of which is modulated by 1,25(OH)2D3 treatment.

Analysis of T-type VSCC α1-subunit transcription levels are displayed in Fig. 3. Expression of Cav3.1 α1G-subunit mRNA was not significantly different in untreated, vehicle-treated, or 0.1 nM 1,25(OH)2D3-treated cells (Fig. 3). When the concentration of 1,25(OH)2D3 was increased to 1 nM, transcription levels were reduced to 48% of the levels measured in vehicle-treated cell cultures. This suppression also was observed when secosteroid levels were increased to 10 nM, and expression was further reduced to 34% of the levels in vehicle-treated cells when the 1,25(OH)2D3 dose was raised to 100 nM. A second T-type VSCC α1-subunit found to be expressed in osteoblastic cells is Cav3.2 (α1H). The expression of Cav3.2 (α1H) transcription levels in ethanol-treated MC3T3-E1 cells was not different from the levels observed in untreated cells (Fig. 3); however, the application of 0.1 nM 1,25(OH)2D3 led to Cav3.2 α1H-subunit transcription levels that were 3.6-fold greater than those found in vehicle-treated MC3T3-E1 cells. This response was maintained when the hormone levels were elevated to 1, 10, and 100 nM 1,25(OH)2D3. The expression of the third T-type VSCC α1-subunit, which we found to be present in osteoblastic cells (Cav3.3 α1I-subunit), was not significantly affected by 1,25(OH)2D3 treatment (Fig. 3).

In addition to L- and T-type VSCCs, MC3T3-E1 cells expressed low levels of mRNA encoding P/Q-, N-, and R-type VSCCs (Fig. 4 and Table 2). The P/Q-type VSCC transcription level encoding Cav2.1 (α1A) in vehicle-treated MC3T3-E1 cells was not significantly different from that found in untreated cells (Fig. 4). Application of 1,25(OH)2D3 for 24 h did not alter Cav2.1 (α1A) mRNA levels when applied at doses of 0.1, 1, or 10 nM (Table 2); however, treatment of MC3T3-E1 cells with a high dose of 1,25(OH)2D3 (100 nM) increased Cav2.1 (α1A) transcription levels 2.2-fold compared with lev-
**P/Q-, N- and R-type channels**

![Graph showing changes in relative P/Q-, N- and R-type VSCC (Voltage-sensitive calcium channels) expression in MC3T3-E1 cells treated with 1,25(OH)2D3.](image)

**Table 2. Transcript levels of VSCC α-subunits in MC3T3-E1 cells treated with 1,25(OH)2D3 (10 nM) or vehicle for 24 h**

<table>
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<th>Subunit</th>
<th>Vehicle, aM</th>
<th>ng of Reverse-Transcribed RNA</th>
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<td>Cav1.3 (α1D)</td>
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<td>Cav1.4 (α1R)</td>
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VSCC, L-type voltage-sensitive Ca\(^{2+}\) channel; 1,25(OH)2D\(_3\), 1,25-dihydroxyvitamin D\(_3\).
**Fig. 5. Immunohistochemistry of VSCC α1-subunits in MC3T3-E1 cells in response to 24-h treatment with 1,25(OH)₂D₃.** Cells were fixed in 4% (vol/vol) paraformaldehyde and stained with rabbit anti-α₁ antibodies. Green staining shows α₁-subunits, and cells were counterstained with the nucleic acid dye ToPro-3 (red). A, C, and E: staining of MC3T3-E1 cells with rabbit anti-Cav1.2 (α₁C) (A), anti-Cav1.3 (α₁D) (C), and anti-Cav3.2 (α₁H) (E), and FITC-conjugated donkey anti-rabbit IgG as secondary antibody. B, D, and F: 24-h 1,25(OH)₂D₃-treated MC3T3-E1 cells stained with rabbit anti-Cav1.2 (α₁C) (B), anti-Cav1.3 (α₁D) (D), anti-Cav3.2 (α₁H) (F), and FITC-conjugated donkey anti-rabbit IgG as secondary antibody. G and H: MC3T3-E1 cells incubated with competing peptides for anti-Cav1.2 (α₁C) (G) and anti-Cav3.2 (α₁H) (H) antibodies. All cultures incubated with the competing peptide or normal rabbit serum showed little green staining and comparable levels of nuclear staining. Note the decrease in Cav1.2 (α₁C) and the increase in Cav3.2 (α₁H) immunostaining in cells treated for 24 h with 1,25(OH)₂D₃.

Additional specificity was demonstrated using preimmune antibodies and, in the case of Cav3.2 (α₁H), by demonstrating negative immunostaining in tissues from mice null for this subunit (41).

**1,25(OH)₂D₃ alters the sensitivity of plasma membrane Ca²⁺ permeability to pharmacological inhibitors.** ⁴⁵Ca²⁺ influx assays were performed to determine which families of VSCCs contribute to osteoblastic cell plasma membrane Ca²⁺ permeability and to determine whether 24-h exposure to 1,25(OH)₂D₃ modulates influx through different VSCC families. MC3T3-E1 cells were pretreated for 15 min with specific VSCC inhibitors, the L-type inhibitor nifedipine (1 μM), the T-type inhibitor sFTX-3.3 (200 nM), the P/Q-type inhibitor ω-agatoxin IVA (1 μM), the N-type inhibitor ω-conotoxin GVIA (1 μM), or vehicle. The cells then were rinsed in HBSS and immediately placed in either resting buffer or depolarizing buffer containing fresh VSCC inhibitors or vehicle. As shown in Fig. 6A, vehicle-treated cells depolarized with the high-K⁺ stimulus demonstrated 3.8-fold greater ⁴⁵Ca²⁺ influx than cells placed in the nondepolarizing resting buffer. Cells treated with nifedipine placed in stimulating buffer displayed a reduction of ⁴⁵Ca²⁺ influx to 37% of vehicle-treated cells in the same buffer, consistent with a major role for this channel previously demonstrated in preosteoblasts (9). Cells treated with inhibitors of T-, P/Q-, and N-type VSCCs had a reduction in ⁴⁵Ca²⁺ influx to 47%, 49%, and 67%, respectively.

MC3T3-E1 cells next were treated for 24 h with 1,25(OH)₂D₃ to determine whether hormone treatment affected cell Ca²⁺ permeability assessed on the basis of sensitivity to class-specific pharmacological inhibition. MC3T3-E1 cells were transferred to serum-free medium for 24 h before being treated with 1,25(OH)₂D₃ or vehicle. Vehicle-treated cells depolarized with high-K⁺ buffer demonstrated a 2.4-fold increase in ⁴⁵Ca²⁺ influx compared with vehicle-treated cells incubated in nondepolarizing resting buffer (Fig. 6B). Total stimulated influx was reduced (from 3.8- to 2.4-fold) in hormone-treated compared with untreated cells, suggesting a slight reduction in total VSCC function after 1,25(OH)₂D₃ treatment. This occurs during the same period and under the same conditions in which we and others have shown that expression of well-established osteoblast differentiation markers, including alkaline phosphatase, osteopontin, and osteocalcin, occur. In MC3T3-E1 cells treated for 24 h with 1,25(OH)₂D₃, blocking the T-type VSCCs reduced ⁴⁵Ca²⁺ influx to 24% of the levels of cells that were not exposed to VSCC inhibitors. Inhibition of P/Q- and L-type VSCCs modestly reduced ⁴⁵Ca²⁺ influx in depolarized 24-h 1,25(OH)₂D₃-treated MC3T3-E1 cells, whereas inhibition of N-type VSCCs had an insignificant effect. Thus treatment of MC3T3-E1 cells with 1,25(OH)₂D₃ for 24 h changes both the pattern of VSCC α₁-subunit transcript expression and the pharmacological sensitivity of the cells to class-specific VSCC inhibitors.

**DISCUSSION**

1,25(OH)₂D₃ is a calcitropic hormone that serves as a key regulator of bone remodeling, and it is well accepted that vitamin D status plays a major role in bone homeostasis. Rapid actions of 1,25(OH)₂D₃ on cells of the osteoblastic lineage include modulation of cAMP (16), phosphatidylinositol 3-ki-
nase (38), activation of Ca^{2+} channels (9), and, along with parathyroid hormone, elevation in the levels of intracellular Ca^{2+} (7, 14, 26). In the longer term, 1,25(OH)_{2}D_{3} also alters rates of gene transcription of target molecules, including markers of osteoblast differentiation (22, 32, 33). This study examined the effect of 24-h 1,25(OH)_{2}D_{3} treatment on patterns of VSCC expression and function in osteoblasts, with the aim of linking rapid and long-term calcemic actions of 1,25(OH)_{2}D_{3} into a novel mechanism describing Ca^{2+} permeability and Ca^{2+}-driven cross talk.

It has been established that depolarization and hormone-stimulated Ca^{2+} influx into osteoblastic cells is inhibited by L-type VSCC inhibitors, including the dihydropyridines (9), and by a ribozyme specifically targeting the L-type VSCC Cav1.2 (α_{1C}) (27). The identification of VSCC transcripts that are typically observed in excitable tissues, including neurons (36) and skeletal muscle (44), in osteoblastic cells is not particularly surprising, given that VSCCs have been found in a variety of nonexcitable tissues and cells, including lung (6), kidney (46), pancreas (40), and fibroblasts (42). In this systematic study of 10 VSCC types, we have shown that although three of the four known L-type VSCCs, Cav1.1 (α_{1S}), Cav1.2 (α_{1C}), and Cav1.3 (α_{1D}), were detected using QPCR in preosteoblastic MC3T3-E1 cells, only Cav1.2 (α_{1C}) was expressed at significant levels. In a previous study, it was shown that application of 1,25(OH)_{2}D_{3} for a duration of seconds to minutes increased the mean open time and Ca^{2+} permeability of the L-type VSCC in the short term (9, 45). In primary osteoblast cultures, 1,25(OH)_{2}D_{3} treatment produces an increase in Ca^{2+} entry that, along with subsequent release of Ca^{2+} from intracellular stores (14, 26), elevates cytoplasmic Ca^{2+} levels. This phenomenon supports signal propagation and stimulates Ca^{2+}-dependent signaling pathways. In addition, Ca^{2+} influx through L-type VSCCs in response to membrane depolarization activates the cAMP- and Ca^{2+}-dependent transcription factor CREB (2). This method of transcriptional activation is most efficient when the Ca^{2+} signal is generated through the L-type VSCC rather than by other methods of Ca^{2+} entry (10). It is thus of critical importance to the function of a cell to modulate both the type and the density of VSCCs expressed on the cell surface.

Using an analog of 1,25(OH)_{2}D_{3} that binds the nuclear vitamin D receptor and does not elicit a plasma membrane-initiated response, we previously demonstrated that the downregulation of the Cav1.2 α_{1C}-subunit involves transcriptional changes that require the presence of the nVDR (30). Consistent with previous studies conducted at our laboratory in which another cell line was used (30), we found in the present study that long-term exposure of MC3T3-E1 cells to nanomolar concentrations of 1,25(OH)_{2}D_{3} decreased Cav1.2 (α_{1C}) mRNA transcription and protein levels. Because mRNA levels for the two other L-type VSCCs detected in osteoblasts, Cav1.3 (α_{1D}) and Cav1.1 (α_{1S}), remain unchanged, it is likely that the Cav1.2 α_{1C}-subunit is the only pore-forming subunit in the...
L-type VSCC family whose transcriptional expression is modulated by 1,25(OH)2D3 treatment. Radioactive Ca2+ influx assays revealed that prolonged exposure to 1,25(OH)2D3 decreased Ca2+ entry through the L-type VSCC, presumably because of decreased expression of the Cav1.2 α1c-subunit. A potential role for downregulation of the Cav1.2 α1c-subunit in response to long-term exposure to 1,25(OH)2D3 is to protect the cell from chronic elevation in intracellular Ca2+ levels that could lead to cell apoptosis. To that end, it has been demonstrated that neuronal vulnerability to excitotoxicity in hippocampal neurons is mediated by Ca2+ influx through the L-type VSCC and that downregulation of these channels with long-term exposure to 1,25(OH)2D3 increases neuroprotection (8). Together, these results suggest that the initial 1,25(OH)2D3 exposure elicits a rapid cellular response, including the activation of various protein kinases, protein lipases, and cAMP, by increasing the short-term ability of Ca2+ to enter the osteoblast through the Cav1.2 α1c-subunit of the L-type VSCC. Long-term exposure to the steroid subsequently downregulates the Cav1.2 α1c-subunit in a nuclear receptor-dependent pathway that then diminishes Ca2+ influx, preventing Ca2+ toxicity.

Although the L-type VSCCs are the best studied Ca2+ channel type and the most highly expressed VSCC in osteoblasts, it has been reported that T-type VSCCs are expressed in osteoblasts during development (13, 29, 41). T-type channels are expressed throughout the body, including in various parts of the nervous system, heart, kidney, smooth muscle, and sperm. These channels have been implicated in a range of physiological events, including cardiac pacemaker activity, neuronal firing, smooth muscle contraction, fertilization, and hormone secretion (34). Electrophysiological properties of T-type currents are similar in many tissues, but differences in inactivation kinetics and pharmacology have been identified and are due to the existence of three distinct T-type VSCCs (15, 24). We report that all three of the T-type VSCC isoforms, Cav3.1 (α1G), Cav3.2 (α1H), and Cav3.3 (α1I), are detectable by performing QPCR in the preosteoblast, but that Cav3.1 (α1G) is the major transcript (Table 2). At the protein level, Cav3.2 (α1H) was prominent at the plasma membrane (Fig. 5), unlike Cav3.1 (α1G), which appeared to be largely intracellular (data not shown). Upon application of 1,25(OH)2D3, Cav3.1 (α1G) transcription levels are reduced by 24 h, whereas the expression of Cav3.2 (α1H) increases almost threefold during the same period. The levels of Cav3.3 (α1I) transcription remain low and unchanged. Immuno staining for Cav3.2 (α1H) revealed a significant increase in the amount of Cav3.2 (α1H) protein in the osteoblastic cells after 24-h treatment with 1,25(OH)2D3. Little staining of Cav3.1 (α1G) protein was observed under any conditions, indicating that the levels of transcription vastly exceeded the amount of protein for this channel class in MC3T3-E1 cells (data not shown), which may be due to a failure to be translated or to assemble. Long-term treatment with 1,25(OH)2D3 concomitantly produces a shift in the proportion of Ca2+ influx into the cells that occurs through the class of T-type VSCCs assessed by susceptibility to pharmacological class-specific blockers. Together, these data suggest that the increases in the Cav3.2 α1H-subunit transcription and protein expression also increase T-type plasma membrane VSCC activity.

Much of the current understanding of the physiological roles of the T-type currents comes from work performed in neuronal (28) and cardiac cells (3), in which the T-type VSCCs generate low-threshold Ca2+ spikes. These Ca2+ spikes are associated with burst firing and oscillatory behavior (23), which produce pacemaker currents and rapid neuronal depolarization. Mice null for the T-type VSCC Cav3.2 (α1H) are viable but show defects in the relaxation of coronary arterioles and focal coronary fibrosis as well as skeletal defects (Ref. 12 and Shao Y, Chen CC, Campbell K, and Farach-Carson MC, unpublished data). The role of the T-type VSCC in the osteoblast has not been studied extensively, although it has been known for some time that MC3T3-E1 cells have T-type currents (1). After treatment with ATP, osteocytic MLO-Y4 cells express T-type currents and Cav3.1 (α1G) transcripts, but Cav3.2 (α1I) levels have not been investigated (21). We recently reported that Cav3.2 α1I-subunits were present in, but Cav1.2 α1C-subunits were absent from, osteocytes in long bone (41). A potential reason for the upregulation of the T-type VSCC Cav3.2 α1I-subunit after treatment with 1,25(OH)2D3 is to compensate for the loss of the T-type VSCC-mediated Ca2+ influx. With the decrease in Cav1.2 (α1C) levels, the ability of the cell to maintain Ca2+-dependent cellular processes would be compromised if a parallel increase in another α1-subunit did not occur. For the terminally differentiated osteocyte, loss of Cav1.2 (α1C) without upregulation of another subunit would render the cell unable to respond to Ca2+-mobilizing stimuli, including mechanical load and shear stress.

N-, P/Q-, and R-type VSCCs typically are found in neuronal cells and are key regulators of neurotransmitter release (11). In osteoblasts, mRNA encoding for Cav2.1 (α1A), Cav2.2 (α1B), and Cav2.3 (α1I) all were detected at low levels. However, application of 1,25(OH)2D3 at physiologically relevant concentrations did not affect mRNA or protein levels for these subunits, except for Cav2.2 (α1B). The downregulation of Cav2.2 (α1B) mRNA observed after 24-h exposure to the steroid did not result in a significant change in the diffuse immunofluorescent staining pattern for the protein (data not shown). Ca2+ influx experiments showed that the N-type VSCC contribution to plasma membrane Ca2+ permeability was not affected by 1,25(OH)2D3 treatment. Together, these data suggest that the P/Q-, N-, and R-type VSCCs are not major contributors to the modulation of osteoblast Ca2+ permeability after 1,25(OH)2D3 treatment.

This study was undertaken to identify the role of VSCCs in regulating Ca2+ permeability in osteoblastic cells under various conditions of vitamin D hormone status. To identify the potential role of the VSCC in Ca2+ permeability, we first had to systematically determine which VSCC α1-subunits are present under various conditions. Although researchers at several laboratories, including our own, have examined the ability of 1,25(OH)2D3 to modulate VSCC activity, a complete explanation of the link between 1,25(OH)2D3 treatment, total VSCC expression, and Ca2+ permeability in the osteoblast has never been reported. A novel aspect of the quantitative approach presented herein is that it allowed us to predict the total potential contribution of L-type and T-type Ca2+ channels to overall Ca2+ permeability under both 1,25(OH)2D3 replete and depleted conditions. The L-type VSCC has a conductance of ~25 pS, whereas the T-type VSCC has a conductance of ~8 pS; thus most Ca2+ entry is due to the amount of functional L-type channel present. The expression levels thus predict that in the absence of vitamin D hormone, cells have approximately
twice the L-type current potential of 1,25(OH)2D3-treated cells. This prediction was borne out in the responses to high K+ concentration in vehicle- and 1,25(OH)2D3-treated cells as shown in Fig. 6. In hormone-treated cells, expression levels of total L-type and T-type VSCC transcripts were reduced to one-half and one-third, respectively (Table 2). However, the increase in the T-type channel Cav3.2 (α1H) expression after hormone treatment was dramatic, especially at the protein level, and likely contributed to the shift to a T-type sensitivity shown in Fig. 6B. In a physiological context, we would suggest that the high Ca2+ permeability of the preosteoblast supports cell proliferation and growth, whereas the loss of Ca2+ permeability during 1,25(OH)2D3-stimulated differentiation is likely to turn off Ca2+-dependent proliferation signals and associated gene expression. Such modulation may occur in parallel with the loss of L-type Cav1.2 (α1C) expression that we recently reported in terminally differentiated osteocytes in intact bone (41).

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REFERENCES


41. Shao Y, Alicknavitch M, and Farach-Carson MC. Expression of voltage sensitive calcium channel (VS CC) L-type CaV1.2 (α1C) and T-type CaV3.2 (α1H) subunits during mouse bone development. *Dev Dyn* 234: 54–62, 2005.


