Down-regulation of L-type Ca$^{2+}$ Channel Transcript Levels by 1,25-Dihydroxyvitamin D$_3$

OSTEOBLASTIC CELLS EXPRESS L-TYPE $\alpha_{1C}$ CA$^{2+}$ CHANNEL ISOFORMS*

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Osteoblast Ca$^{2+}$ channels play a fundamental role in controlling intracellular and systemic Ca$^{2+}$ homeostasis. A reverse transcription-polymerase chain reaction strategy was used to determine the molecular identity of voltage-sensitive calcium channels present in ROS 17/2.8 osteosarcoma cells. The amino acid sequences encoded by the two resultant PCR products matched the $\alpha_{1C}$ and the $\alpha_{1A}$ isoforms. The ability of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) and structural analogs to modulate expression of voltage-sensitive calcium channel mRNA transcripts was then investigated. ROS 17/2.8 cells were cultured for 48 h in the presence of either 1,25(OH)$_2$D$_3$, 1,24-dihydroxy-22-ene-24-cyclopropyl-D$_3$ (analog BT) or 25-hydroxy-16-ene-23-yne-D$_3$ (analog AT), and the levels of mRNA encoding $\alpha_{1C}$ were quantitated using a competitive reverse transcription-polymerase chain reaction assay. We found that 1,25(OH)$_2$D$_3$ and analog BT reduced steady state levels of $\alpha_{1C}$ mRNA. Conversely, the Ca$^{2+}$-stimulating analog AT did not alter steady state levels of voltage-sensitive calcium channel mRNA. Since analog BT, but not analog AT, binds and transcriptionally activates the nuclear receptor for 1,25(OH)$_2$D$_3$, these findings suggest that the down-regulation of voltage-sensitive calcium channel mRNA levels may involve the nuclear receptor.

The balance between osteoclastic bone resorption and osteoblastic bone formation determines skeletal mass and composition. 1,25-Dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) has long been appreciated as a hormonal modulator of osteoblast function and bone remodeling. 1,25(OH)$_2$D$_3$, classically considered a resorptive hormone, has the paracrine effects on osteoclasts and the levels of mRNA encoding receptor for 1,25-dihydroxyvitamin D$_3$; RT, reverse transcription; PCR, polymerase chain reaction; analog BT, 1,24-(OH)$_2$-22-ene-24-cyclopropyl-D$_3$; analog AT, 25(OH)-16-ene-23-yne-D$_3$; bp, base pairs.

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1 The abbreviations used are: 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; VSCC(s), voltage-sensitive calcium channel(s); nVDR, nuclear receptor for 1,25-dihydroxyvitamin D$_3$; RT, reverse transcription; PCR, polymerase chain reaction; analog BT, 1,24-(OH)$_2$-22-ene-24-cyclopropyl-D$_3$; analog AT, 25(OH)-16-ene-23-yne-D$_3$; bp, base pairs.

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blastic cells has not been previously investigated. Additionally, because the extent and ability of 1,25(OH)\textsubscript{2}D\textsubscript{3} to rapidly increase plasma membrane Ca\textsuperscript{2+} permeability is directly related to the density of functional VSCCs, we investigated the long term ability of 1,25(OH)\textsubscript{2}D\textsubscript{3} to modulate transcript levels encoding the \( \alpha \) subunit of the VSCC.

In this report, partial cDNA cloning of the L-type VSCC \( \alpha \) subunit in ROS 17/2.8 osteoblastic cells is presented. Nondegenerate reverse transcription-polymerase chain reaction (RT-PCR) primers were designed to amplify and sequence the IV S3-IV S4 region of the channel, an isoform-specific region. Amplimer sequencing demonstrated that the deduced amino acid sequence of the \( \alpha \) subunit is homologous to two VSCC isoforms, \( \alpha \text{1C-a} \) and \( \alpha \text{1C-d} \). Furthermore, quantitative PCR was used to study the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and analogs on the levels of L-type VSCC mRNA. It was found that 1,25(OH)\textsubscript{2}D\textsubscript{3} down-regulates the \( \alpha \text{1C} \) transcript levels at a physiological dose (1 nM), and vitamin D analog studies suggest that this down-regulation involves the vD3R.

EXPERIMENTAL PROCEDURES

Cell Culture—ROS 17/2.8 osteosarcoma cells were maintained in culture as described previously (10). Primary rat calvarial osteoblasts were isolated by sequential enzymatic digestion (21). Cells were then grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. For mRNA quantitation, cells were passaged 1:10 and grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. For mRNA quantitation, cells were passaged 1:10 and grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. For mRNA quantitation, cells were passaged 1:10 and grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. For mRNA quantitation, cells were passaged 1:10 and grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

RT-PCR Primer Selection for Cloning—The PCR primers were selected based on conserved regions of the \( \alpha \) subunit such that nonconserved, isoform-specific sequences would be amplified during the reaction. The PCR primers were designated as PR 1/PR 2 as reported previously (1). Briefly, cells were washed twice with ice-cold balanced buffer to assess fragment size. The PR 1/PR 2 amplifier was then ligated into the PCR2000 vector (TA Cloning, San Diego, CA) using T4 ligase, and this mixture used to transform Escherichia coli (23). Plasmid DNA was isolated from the cultures using an alkaline lysis method and digested with EcoRI to confirm the presence of the desired insert (~900 bp). Inserts were sequenced using the Sequenase Version 2.0 kit (U. S. Biochemical Corp.).

Quantitative of VSCC Transcript Levels by RT-PCR—For quantitation of VSCC mRNA levels, a pair of primers designed to amplify a 246-bp region of the \( \alpha \) subunit was chosen, designated as 2514/2759, and used previously by Iwashima et al. (24). An internal standard cDNA for competitive quantitative PCR was generated using PCR with an upstream primer of 40 bp designed to anneal to two 20-bp regions of the \( \alpha \) subunit located 100 bp apart. PCR under low stringency conditions (50 °C annealing) produced a cDNA containing sequences identical with those of the target (VSCC) cDNA, each of which is recognized by both +2514 and −2759 primers, thus serving as an appropriate competitor to the endogenous target. The competitor cDNA (denoted M146) and the target cDNA are easily distinguished by size. PCR reactions using 20 ng of RNA template were performed for 35 cycles in the presence of serial dilutions of M146 (0.05–5.0 fg) and 2 μCi (a-32P)CTP using the following conditions: 30 s at 94 °C for denaturation; 1 min at 60 °C for primer annealing; and 2 min at 72 °C for polymerization. Products were separated by electrophoresis on 5% polyacrylamide gels which were dried and exposed to film for 24 h.

RESULTS

PCR Amplification—We selected sequences within the \( \alpha \) subunit of L-type VSCCs as the target for PCR amplification based on the extensive sequence data available in the literature. This region was carefully selected to include conserved and nonconserved sequences of various isoforms including those from rabbit cardiac muscle, skeletal muscle, neuroendocrine tissue, and brain. The sequence of each primer used in this study is listed in Table I. The amplification products of the PR 1 and PR 2 primers produced from ROS 17/2.8 cells are shown in Fig. 1, which shows two bands, one of 903 bp and one of ~870 bp; the identity of each is discussed below.

Quantitation of VSCC mRNA Levels following Exposure to...
amino acid sequence of each product is shown below (see Fig. 2).

Additional, the ROS 17/2.8 VSCCs contain a 33-bp deletion which gives rise to the alternatively spliced isoform, α1C-d. The deletion is shown by a series of periods below the full length sequence in bold face. Dashes indicate matches with the α1C-a sequence, and the location of the sequence within the putative structure of the α1 subunit is shown in brackets above the sequence. TM, transmembrane.

1,25(OH)2D3 and Analogs—Quantitative competitive RT-PCR was utilized to quantify the mRNA levels for the α1 subunit following 48 h of exposure to 1,25(OH)2D3, vitamin D analogs or to vehicle (0.1% absolute ethanol) followed by isolation of total RNA. The primers used for PCR were designed to amplify a 246-bp VSCC cDNA (target) and a 146-bp competitor cDNA (M146). It was determined that a suitable linear range for this 35-cycle reaction lies between 10 and 80 ng of target RNA (Fig. 3A). We routinely used 20 ng of RNA and between 0.2 and 3 ng of the competitor cDNA (Fig. 3B) to compare VSCC mRNA levels in each treatment group. The densitometrically measured ratio of competitor/target can be plotted as a function of increasing amounts of competitor; thus, the slope of the regression line is inversely proportional to the mRNA levels.

Treatment with both 1,25(OH)2D3 and analog BT, which binds the nVDR and does not acutely activate plasma membrane Ca2+ influx, lowered mRNA levels of the VSCC α1C-a subunit. The concentrations used for these hormones were 1 nM for 1,25(OH)2D3 and 10 nM for analog BT, concentrations shown in our previous studies to induce maximal Ca2+ uptake and maximal transcription of target genes, respectively (10, 22). Fig. 4 demonstrates the down-regulation of VSCC transcript levels by 1,25(OH)2D3 (Fig. 4A) and analog BT (Fig. 4B) plotted using linear regression analysis of scanned autoradiographs. Based on the point of equivalence, at which the ratio of target to competitor is equal to 1, the relative amounts of VSCC α1C transcript present in the 1,25(OH)2D3- and analog BT-treated cells were 48 and 44% of vehicle control, respectively. As stated in the legend to Fig. 4, these experiments were repeated 5–7 times. The r2 values for all data points available for each treatment group are 0.96 (1,25(OH)2D3); 0.98 (analog BT), and 0.92 (vehicle control). In contrast, analog AT, which preferentially induces maximal Ca2+ uptake at 1 nM without binding to the nVDR, does not alter VSCC mRNA levels relative to control mRNA at this concentration (Fig. 5). In three additional independent experiments, the values for mRNA levels from cells treated with analog AT or vehicle control were not significantly different from each other: vehicle (competitor/target) 2.13, 1.57, 3.06, mean = 2.25 ± 0.75; analog AT (competitor/target) 1.63, 2.08, 2.66, mean = 2.12 ± 0.52.

**DISCUSSION**

We cloned a portion of the cDNA encoding the ROS 17/2.8 L-type VSCC α1 subunit and found it to encode a protein product identical with two isoforms of the cardiac VSCC, α1C-a and α1C-d in the fourth transmembrane region. The α1C isoform was also identified in primary rat calvarial osteoblasts. It is not surprising that both of these isoforms exist in osteoblastic cells, given that they are found in a variety of tissues and cells such as lung (25), aorta (16), brain (19), and fibroblasts (26). While it has been known for some time that various cells of the osteoblastic lineage contain functional Ca2+ channels, the molecular nature of these channels has only recently been studied in ROS 17/2.8 cells using an RT-PCR experimental strategy (27). Barry et al. (28) extended these studies to show that UMR-106 osteo-
Down-regulated Osteoblastic Ca\textsuperscript{2+} Channel Transcripts

**FIG. 4.** Down-regulation of \( \alpha_1C \) transcripts by 1,25(OH)\textsubscript{2}D\textsubscript{3} and analog BT. Regression lines generated by quantitative PCR data in which 20 ng of target RNA and 0.2–3.0 fg of competitor cDNA were amplified, quantitated by scanning densitometry, and plotted as a ratio of target to competitor. A, products from ROS 17/2.8 cells treated with 1 ng of 1,25(OH)\textsubscript{2}D\textsubscript{3} (○) for 48 h are plotted against vehicle-treated controls (●). B, the products from cells treated with 10 ng of analog BT (○) for 48 h are compared with controls (●). The increased slope in the 1,25(OH)\textsubscript{2}D\textsubscript{3} and analog BT plots indicate decreased amounts of VSCC \( \alpha_1 \) subunit mRNA present in these samples. In each graph, data points were averaged directly from two individual experiments in which all data points were available. The error bars for these two experiments are contained within the symbols. The trend was also reproduced in five separate experiments, which were not included in this graph because all data points were not available in each series.

FIG. 5. Comparison of vitamin D analogs at maximum effective concentrations. Autoradiograph of quantitative PCR products in which only a single concentration of the competitor, M146 (1.5 fg) was used in amplification reactions containing 20 ng of RNA from controls, 1,25(OH)\textsubscript{2}D\textsubscript{3}, analog BT-1, or analog AT-treated cells. The ratio of products (competitor:target) is listed at the bottom. The concentrations used for treatment of cells for 48 h prior to RNA isolation were 1 nM for 1,25(OH)\textsubscript{2}D\textsubscript{3} and analog AT and 10 nM for analog BT.

Osteoblasts in their bone microenvironment is the ability to regulate the secretion of various matrix proteins in response to hormonal and mechanical stimuli.

Over the past decade, 1,25(OH)\textsubscript{2}D\textsubscript{3} has emerged as an important hormone in the physiology of many tissues. While generally considered a resorptive hormone in bone, it has anabolic effects on the expression of many extracellular matrix proteins that contribute to bone mass. In fact, the majority of the osteoblasts in their bone microenvironment is the ability to regulate the secretion of various matrix proteins in response to hormonal and mechanical stimuli.

**In summary,** this study provides evidence for the identity of L-type Ca\textsuperscript{2+} channel isoforms (\( \alpha_1C \)) in osteoblastic cells. Furthermore, the demonstration that 1,25(OH)\textsubscript{2}D\textsubscript{3} down-regulates the steady state levels of mRNA encoding VSCCs points out the interdependence of the rapid, membrane-initiated, and sarcolemmal cells contain skeletal (\( \alpha_{1S} \)), cardiac (\( \alpha_{1C} \)), and neuroendocrine (\( \alpha_{1N} \)) isoforms of the L-type Ca\textsuperscript{2+} channel in a small region of the \( \alpha_1 \) cDNA. With regard to \( \alpha_{1C} \), we find evidence for two cardiac isoforms, \( \alpha_{1C-a} \) and \( \alpha_{1C-d} \) in ROS 17/2.8 cells. Interestingly, it has been reported that when UMR-106 cells are subjected to chronic mechanical strain, they acquire a calcium conductance that responds to hypotonic swelling and can be blocked by antisense RNA to \( \alpha_{1C} \) (29). Thus, regulation of Ca\textsuperscript{2+} channel expression in osteoblasts may be complex, being affected by hormonal and mechanical interactions as well as the stage of cell differentiation (30).

Osteoblasts are generally considered to be nonexcitable cells that share few characteristics with excitable cells in skeletal and cardiac muscle. With regard to plasma membrane Ca\textsuperscript{2+} permeability, an obvious difference between osteoblasts and the excitable tissues is the total number of the functional VSCCs expressed. ROS 17/2.8 cells express between 1000 and 2000 functional L-type Ca\textsuperscript{2+} channels per cell (5) while differentiated myocytes contain at least 10 times this level (31). This difference in VSCC levels is also apparent at the mRNA level, in which standard Northern blotting techniques used routinely for excitable tissues are frequently incapable of detecting low levels of VSCC mRNA in cells such as ROS 17/2.8. Given our findings that osteoblasts express VSCC isoforms common to cardiac tissues, osteoblasts may represent a “hybrid” between excitable and nonexcitable cells that may possess unique characteristics that relate to function. One such function of osteoblasts in their bone microenvironment is the ability to regulate the secretion of various matrix proteins in response to hormonal and mechanical stimuli.

**In summary,** this study provides evidence for the identity of L-type Ca\textsuperscript{2+} channel isoforms (\( \alpha_{1C} \)) in osteoblastic cells. Furthermore, the demonstration that 1,25(OH)\textsubscript{2}D\textsubscript{3} down-regulates the steady state levels of mRNA encoding VSCCs points out the interdependence of the rapid, membrane-initiated, and
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genomic effects of this hormone. By affecting the biosynthesis of the VSCCs at a genomic level, 1,25(OH)$_2$D$_3$ can alter membrane permeability to Ca$^{2+}$ and thus the capacity of the osteoblast to respond to a variety of hormones unique to bone physiology.

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