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Short Communication

1α,25-dihydroxyvitamin D₃ Rapidly Modulates Ca²⁺ Influx in Osteoblasts Mediated by Ca²⁺ Channels

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Abstract

The biologically active form of vitamin D, 1α,25-dihydroxy vitamin D₃ (VD), regulates the synthesis of the bone Ca-binding proteins osteocalcin and osteopontin. The actions of VD are mediated through the vitamin D receptor (VDR). Liganded VDR heterodimerizes with the retinoid X receptor and interacts with a vitamin D response element (VDRE). Recently, it has been demonstrated that vitamin D responses elicited in osteoblasts can be rapid as well as long-term. The purpose of this study was to elucidate the mechanism of Ca²⁺ signaling of VD in osteoblasts using intracellular Ca²⁺ ([Ca²⁺]ᵢ) measurements. A rapid VD (10 nM)-induced increase in [Ca²⁺]ᵢ was observed within 40 sec. This increase, however, was negated with application of Ca²⁺-free Krebs’ solution. These results indicate that VD induces an increase in [Ca²⁺]ᵢ from extracellular Ca²⁺ in osteoblasts.

Key words: Non-genomic action—1α,25-dihydroxyvitamin D₃—Osteoblasts

Introduction

Osteoblasts play a major role in bone formation. Osteoblasts employ intracellular Ca²⁺ ([Ca²⁺]ᵢ) as a second messenger modulating hormonal responses and also a cofactor for bone mineralization. When sufficient amounts of Ca²⁺ and phosphate are supplied, osteoblasts synthesize and secrete a collagenous extracellular matrix in response to diverse extracellular stimuli such as hormones, growth factors, cytokines, biochemical signals and extracellular Ca²⁺ concentration. 1α,25-dihydroxyvitamin D₃ (VD), the active form of vitamin D³, is a major calcitropic hormone involved in Ca²⁺ homeostasis. One of its functions in bone is to regulate the synthesis of the bone Ca²⁺-binding proteins osteocalcin and osteopontin. In addition, VD regulates cell proliferation and differentiation via Ca²⁺ signals in osteoblasts.

Expression of these genes is modulated by VD through transcriptional regulation. The actions of VD are mediated through the vitamin D receptor (VDR). Liganded VDR heterodimerizes with the retinoid X receptor and interacts with a vitamin D response element (VDRE).
Recently, it has been demonstrated that rapid responses are elicited in osteoblasts\textsuperscript{14,21}. The purpose of this study was to elucidate the mechanism of Ca\textsuperscript{2+} signaling of VD in osteoblasts using [Ca\textsuperscript{2+}]\textsubscript{i} measurements.

**Materials and Methods**

1. **Cell culture**

Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in a 5% CO\textsubscript{2} atmosphere with α-modified minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY, U.S.A.). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cell culture medium was changed every 2–3 days. For free [Ca\textsuperscript{2+}]\textsubscript{i} measurements, the cells were plated in 10 tissue culture dishes and grown until reaching confluence.

When the cells reached confluence, they were harvested using a 0.05% trypsin—0.02% EDTA solution for [Ca\textsuperscript{2+}]\textsubscript{i} measurements. Harvested cells were then plated at very low density in 35-mm tissue culture dishes and grown to 75% confluence. Prior to recordings, the cells were washed at least 3 times with electrophysiological external solution to remove the medium completely. Cell culture reagents and most other biochemical reagents were purchased from Sigma Chemical Co. (MO, U.S.A.) unless otherwise specified.

2. **[Ca\textsuperscript{2+}]\textsubscript{i} measurements in osteoblasts**

[Ca\textsuperscript{2+}]\textsubscript{i} was measured in osteoblasts using fura-2 applied to microfluorescent digital video imaging\textsuperscript{10}. The 75% confluent cells were loaded with 4 μM fura-2/AM and 15% Pluronic F-127 (Molecular Probes, Eugene, OR) for 90 min at room temperature. [Ca\textsuperscript{2+}]\textsubscript{i} was measured in fura-2-loaded cells in Krebs’ solution buffer containing (in mM) 138.5 NaCl, 5 KCl, 1.1 NaH\textsubscript{2}PO\textsubscript{4}, 2H\textsubscript{2}O, 11.9 NaHCO\textsubscript{3}, 0.5 MgCl\textsubscript{2}, 6H\textsubscript{2}O, and 10.9 glucose. Ratio images (340–380 nm excitation, 510 nm emission) were collected every 5 sec\textsuperscript{10}. The ratio analysis was then transformed into F/F\textsubscript{0} values, which means that any fluorescence readings during stimulation (F) were referred to the reading at rest (F\textsubscript{0}), i.e., at time t = 0 just before stimulation.

3. **Chemicals**

The VD, which was purchased from Biomol Research Laboratories (Plymouth, PA, U.S.A.), was dissolved in ethanol.

The final concentration of ethanol was <0.01%, which had no effect on [Ca\textsuperscript{2+}]\textsubscript{i} measurements.

**Results**

1. **VD increases [Ca\textsuperscript{2+}]\textsubscript{i} in MC3T3-E1 rapidly**

To investigate whether VDR activation elicited [Ca\textsuperscript{2+}]\textsubscript{i} signals in MC3T3-E1 cells, we first
stimulated the cells with VD (10 nM, 60 sec). As shown in Fig. 1, the VD induced a transient increase in $[\text{Ca}^{2+}]_{i}$ that reached a maximum within 40 sec after VD addition and returned to baseline within 30–40 sec. This rapid response suggests that VD promotes nongenomic action.

Whereas the onset of $[\text{Ca}^{2+}]_{i}$ signaling is induced by $[\text{Ca}^{2+}]_{i}$ release from intracellular stores, VD response depends on the amount of $[\text{Ca}^{2+}]_{i}$ entering the cytoplasm, both from stores and extracellular fluid.

2. Extracellular $[\text{Ca}^{2+}]_{i}$ is source of increased $[\text{Ca}^{2+}]_{i}$, induced by VD interaction

In osteoblasts, $[\text{Ca}^{2+}]_{i}$, is provided by $[\text{Ca}^{2+}]_{i}$ release through the inositol-1,4,5-phosphate and ryanodine receptors of endoplasmic reticulum and $[\text{Ca}^{2+}]_{i}$ influx via $[\text{Ca}^{2+}]_{i}$ channels or store-operated $[\text{Ca}^{2+}]_{i}$ influx from extracellular $[\text{Ca}^{2+}]_{i}$.

To investigate the source of the $[\text{Ca}^{2+}]_{i}$, response to VD, MC3T3-E1 cells were washed for several seconds with $[\text{Ca}^{2+}]_{i}$-free Krebs’ solution immediately before VD treatment. Depletion of extracellular $[\text{Ca}^{2+}]_{i}$ with $[\text{Ca}^{2+}]_{i}$-free Krebs’ solution in this way completely blocked the increase in $[\text{Ca}^{2+}]_{i}$, otherwise induced by VD, consistent with a mechanism involving influx of extracellular $\text{Ca}^{2+}$ (Fig. 2).

These results indicated that VD induced $[\text{Ca}^{2+}]_{i}$ increases from extracellular $\text{Ca}^{2+}$ in osteoblasts.

Discussion

The present study investigated the effects of VD on $[\text{Ca}^{2+}]_{i}$, in osteoblasts. Application of VD rapidly increased $[\text{Ca}^{2+}]_{i}$, and VD-induced $[\text{Ca}^{2+}]_{i}$ increases were undetectable in the absence of extracellular $\text{Ca}^{2+}$.

In bone, VD is responsible for increased transcription of bone matrix proteins such as collagen type I, osteocalcin, and osteopontin produced by osteoblasts in the process of bone formation. It is well established that VD stimulates osteoblast growth and differentiation. Bhatia et al. suggested that VD-induced differentiation of promyelocytic leukemia cells implied non-genomic action and second messengers such as PKC and tyrosine kinases. These transcriptional events are evoked by VD binding to retinoid X receptors and interaction with a VDRE. In this study, however, application of VD rapidly increased $[\text{Ca}^{2+}]_{i}$. It seems unlikely that only retinoid X receptors and VDRE act as VDRs.

In osteoblasts, VD promotes two types of action: genomic and nongenomic. Recently, it was demonstrated that nongenomic actions are elicited through a membrane-associated VDR. Voltage-dependent calcium channels have been classified as follows based on their physiological and pharmacological properties: low-voltage activated voltage-dependent calcium channels and high-voltage activated voltage-dependent calcium channels. Additionally, high-voltage activated voltage-dependent calcium channels can be classified as L-, N-, P-, Q- or R-type voltage-dependent calcium channels. L-type voltage-dependent calcium channels exhibit long-lasting and large-conductance channel properties. Several studies have demonstrated that activation of membrane-associated VDR facilitates L-type $[\text{Ca}^{2+}]_{i}$ channels and increases $[\text{Ca}^{2+}]_{i}$ concentration.
addition, it has also been demonstrated that phosphorylation of the L-type Ca\(^{2+}\) channels occurs via protein kinase A activation by VD signaling in osteoblasts\(^{30}\).

In addition to VD, acetylcholine also induces an increase in \([\text{Ca}^{2+}]/\text{H}\) \(\text{Ca}^{2+}\) channels, mediated by muscarinic receptors. Acetylcholine generates an increase in \([\text{Ca}^{2+}]/\text{H}\) with a rapid transient rising phase followed by a slow, long, sustained phase. Muscarinic receptor-activated inositolphospholipid turnover involves a direct coupling of the receptor to inositolphospholipid-specific phospholipase C through G\(_{1}\) protein\(^{2}\). Moreover, phospholipase A\(_{2}\) activation requires the sustained but not transient increase of \([\text{Ca}^{2+}]/\text{H}\) \(11\).

What is the physiological relevance of VD-induced nongenomic action in osteoblasts? One earlier study demonstrated VD-induced exocytotic release of ATP from osteoblasts within 60 sec\(^{7}\). Voltage-dependent calcium channels serve as crucial mediators of exocytotic release, including in neurotransmitters\(^{12,23,31}\). Therefore, it is possible that VD-induced nongenomic action modulates exocytotic release in osteoblasts.

In addition to mediation of VD-induced increase in \([\text{Ca}^{2+}]/\text{H}\) by VDRs, it is also possible that VD activates voltage-dependent calcium channels directly. For example, it has been reported that 1,4-dihydropyridines BAY K 8644 activates L-type voltage-dependent calcium channels in cardiac cells\(^{4}\). Further research is needed to clarify whether VD acts as a voltage-dependent calcium channels activator.

In osteoblastic ROS17/2.8 cells and non-osteoblastic CV-1 cells, VD-induced increase in \([\text{Ca}^{2+}]/\text{H}\), involved PI3K-Akt pathways\(^{32}\). This suggests the need for further study on VD pathways.

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**References**


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