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**Short Communication**

# 1$\alpha$,25-dihydroxyvitamin $D_3$ Rapidly Modulates $Ca^{2+}$ Influx in Osteoblasts Mediated by $Ca^{2+}$ Channels

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## Abstract

The biologically active form of vitamin D, 1$\alpha$,25-dihydroxy vitamin $D_3$ (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 $Ca^{2+}$ signaling of VD in osteoblasts using intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) measurements. A rapid VD (10 nM)-induced increase in $[Ca^{2+}]_i$ was observed within 40 sec. This increase, however, was negated with application of $Ca^{2+}$-free Krebs' solution. These results indicate that VD induces an increase in $[Ca^{2+}]_i$ from extracellular $Ca^{2+}$ in osteoblasts.

Key words: Non-genomic action—1$\alpha$,25-dihydroxyvitamin $D_3$—Osteoblasts

## Introduction

Osteoblasts play a major role in bone formation. Osteoblasts employ intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) as a second messenger modulating hormonal responses and also a cofactor for bone mineralization[15,18]. When sufficient amounts of $Ca^{2+}$ and phosphate are supplied, osteoblasts synthesize and secret a collagenous extracellular matrix in response to diverse extracellular stimuli such as hormones, growth factors, cytokines, biochemical signals and extracellular $Ca^{2+}$ concentration[8,16,33].

1$\alpha$,25-dihydroxyvitamin $D_3$ (VD), the active form of vitamin D[17], is a major calcitropic hormone involved in $Ca^{2+}$ homeostasis. One of its functions in bone is to regulate the synthesis of the bone $Ca^{2+}$-binding proteins osteocalcin and osteopontin. In addition, VD regulates cell proliferation and differentiation[22,28] *via* $Ca^{2+}$ 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[14,21]. The purpose of this study was to elucidate the mechanism of $Ca^{2+}$ signaling of VD in osteoblasts using $[Ca^{2+}]_i$ measurements.

## Materials and Methods

### 1. Cell culture

Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in a 5% $CO_2$ atmosphere with $\alpha$-modified minimal essential medium ($\alpha$-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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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. $[Ca^{2+}]_i$ measurements in osteoblasts

$[Ca^{2+}]_i$ was measured in osteoblasts using fura-2 applied to microfluorescent digital video imaging[10]. The 75% confluent cells were loaded with 4$\mu$M fura-2/AM and 15% Pluronic F-127 (Molecular Probes, Eugene, OR) for 90 min at room temperature. $[Ca^{2+}]_i$ was measured in fura-2-loaded cells in Krebs' solution buffer containing (in mM) 136 NaCl, 5 KCl, 1.1 $NaH_2PO_4 \cdot 2H_2O$, 11.9 $NaHCO_3$, 0.5 $MgCl_2 \cdot 6H_2O$, 2.5 $CaCl_2$ and 10.9 glucose. Experiments performed in the absence of extracellular $Ca^{2+}$ were performed in a buffer of a similar composition to that of the above, that is, $Ca^{2+}$-free Krebs' solution buffer containing (in mM) 138.5 NaCl, 5 KCl, 1.1 $NaH_2PO_4 \cdot 2H_2O$, 11.9 $NaHCO_3$, 0.5 $MgCl_2 \cdot 6H_2O$, and 10.9 glucose. Ratio images (340–380 nm excitation, 510 nm emission) were collected every 5 sec[10]. The ratio analysis was then transformed into F/F0 values, which means that any fluorescence readings during stimulation (F) were referred to the reading at rest (F0), *i.e.*, at time t = 0 just before stimulation.

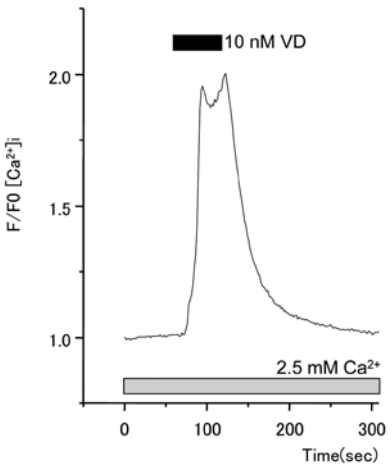


Fig. 1 Effect of VD on intracellular cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) in osteoblast-like cells (MC3T3-E1)

Representative transient increase in $[Ca^{2+}]_i$ in response to fixed dose of VD (10 nM) in MC3T3-E1. *Ordinate*: ratio of fura-2 emission at 340- and 380-nm excitation. *Abscissa*: time of observation (seconds, sec). Addition of VD to MC3T3-E1 is indicated by bar (bar = 60 sec).

### 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 $[Ca^{2+}]_i$ measurements.

## Results

### 1. VD increases $[Ca^{2+}]_i$ in MC3T3-E1 rapidly

To investigate whether VDR activation elicited $[Ca^{2+}]_i$ signals in MC3T3-E1 cells, we first

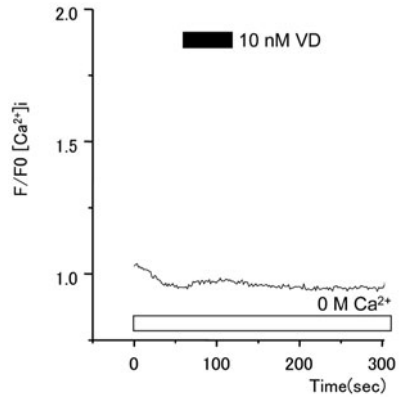


Fig. 2 Source of $Ca^{2+}$ signaling induced by VD interaction in osteoblast-like cells (MC3T3-E1)

*Ordinate*: ratio of fura-2 emission at 340- and 380-nm excitation. *Abscissa*: time of observation (seconds, sec). Addition of VD to MC3T3-E1 in absence of extracellular $Ca^{2+}$ ($[Ca^{2+}]$out = 0 M) is indicated by bar (bar = 60 sec).

stimulated the cells with VD (10 nM, 60 sec). As shown in Fig. 1, the VD induced a transient increase in $[Ca^{2+}]_i$ 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 $Ca^{2+}$ signaling is induced by $Ca^{2+}$ release from intracellular stores[29], VD response depends on the amount of $Ca^{2+}$ entering the cytoplasm, both from stores and extracellular fluid.

### 2. Extracellular $Ca^{2+}$ is source of increased $[Ca^{2+}]_i$ induced by VD interaction

In osteoblasts, $[Ca^{2+}]_i$ is provided by $Ca^{2+}$ release through the inositol-1,4,5-phosphate and ryanodine receptors of endoplasmic reticulum and $Ca^{2+}$ influx *via* $Ca^{2+}$ channels or store-operated $Ca^{2+}$ influx from extracellular $Ca^{2+}$[1,3].

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

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

## Discussion

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

In bone, VD is responsible for increased transcription of bone matrix proteins such as collagen type I[12,27], osteocalcin[19,24] and osteopontin[20,25] produced by osteoblasts in the process of bone formation. It is well established that VD stimulates osteoblast growth and differentiation[30]. 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[5,6]. 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 $[Ca^{2+}]_i$. 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[14,21]. 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[9,26]. Several studies have demonstrated that activation of membrane-associated VDR facilitates L-type $Ca^{2+}$ channels and increases $[Ca^{2+}]_i$ concentration[34]. In

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[34].

In addition to VD, acetylcholine also induces an increase in $[Ca^{2+}]_i$ mediated by muscarinic receptors. Acetylcholine generates an increase in $[Ca^{2+}]_i$, 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 $G_q$ protein[2]. Moreover, phospholipase $A_2$ activation requires the sustained but not transient increase of $[Ca^{2+}]_i$[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[13,23,31]. Therefore, it is possible that VD-induced nongenomic action modulates exocytotic release in osteoblasts.

In addition to mediation of VD-induced increase in $Ca^{2+}$ 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 $[Ca^{2+}]_i$ involved PI3K-Akt pathways[32]. This suggests the need for further study on VD pathways.

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