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Short Communication

## 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> Rapidly Modulates Ca<sup>2+</sup> Influx in Osteoblasts Mediated by Ca<sup>2+</sup> Channels

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### Abstract

The biologically active form of vitamin D, 1 $\alpha$ ,25-dihydroxy vitamin D<sub>3</sub> (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<sup>2+</sup> signaling of VD in osteoblasts using intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) measurements. A rapid VD (10 nM)-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> was observed within 40sec. This increase, however, was negated with application of Ca<sup>2+</sup>-free Krebs' solution. These results indicate that VD induces an increase in [Ca<sup>2+</sup>]<sub>i</sub> from extracellular Ca<sup>2+</sup> in osteoblasts.

Key words: Non-genomic action—1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>—Osteoblasts

### Introduction

Osteoblasts play a major role in bone formation. Osteoblasts employ intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) as a second messenger modulating hormonal responses and also a cofactor for bone mineralization<sup>15,18)</sup>. When sufficient amounts of Ca<sup>2+</sup> 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 Ca<sup>2+</sup> concentration<sup>8,16,33)</sup>.

1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (VD), the active

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

## Materials and Methods

### 1. Cell culture

Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in a 5%  $\text{CO}_2$  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  $[\text{Ca}^{2+}]_i$  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  $[\text{Ca}^{2+}]_i$  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. $[\text{Ca}^{2+}]_i$ measurements in osteoblasts

$[\text{Ca}^{2+}]_i$  was measured in osteoblasts using fura-2 applied to microfluorescent digital video imaging<sup>10</sup>. The 75% confluent cells were loaded with 4  $\mu\text{M}$  fura-2/AM and 15% Pluronic F-127 (Molecular Probes, Eugene, OR) for 90 min at room temperature.  $[\text{Ca}^{2+}]_i$  was measured in fura-2-loaded cells in Krebs' solution buffer containing (in mM) 136 NaCl, 5 KCl, 1.1  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 11.9  $\text{NaHCO}_3$ , 0.5  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.5  $\text{CaCl}_2$  and 10.9 glucose. Experiments performed in the absence of extracellular  $\text{Ca}^{2+}$  were performed in a buffer of a similar composition to that of the above, that is,

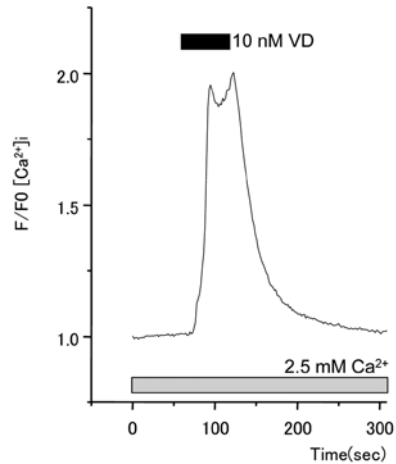


Fig. 1 Effect of VD on intracellular cytosolic  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in osteoblast-like cells (MC3T3-E1)

Representative transient increase in  $[\text{Ca}^{2+}]_i$  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).

$\text{Ca}^{2+}$ -free Krebs' solution buffer containing (in mM) 138.5 NaCl, 5 KCl, 1.1  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 11.9  $\text{NaHCO}_3$ , 0.5  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and 10.9 glucose. Ratio images (340–380 nm excitation, 510 nm emission) were collected every 5 sec<sup>10</sup>. 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.

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

## Results

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

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

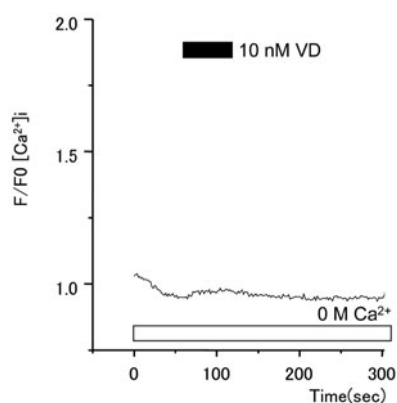


Fig. 2 Source of Ca $^{2+}$  signaling induced by VD interaction in osteoblast-like cells (MC3T3-E1)

*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<sup>29</sup>, 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 $^{2+}$ <sup>1,3</sup>.

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

addition, it has also been demonstrated that phosphorylation of the L-type  $\text{Ca}^{2+}$  channels occurs *via* protein kinase A activation by VD signaling in osteoblasts<sup>34</sup>.

In addition to VD, acetylcholine also induces an increase in  $[\text{Ca}^{2+}]_i$  mediated by muscarinic receptors. Acetylcholine generates an increase in  $[\text{Ca}^{2+}]_i$  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<sup>2</sup>. Moreover, phospholipase  $A_2$  activation requires the sustained but not transient increase of  $[\text{Ca}^{2+}]_i$ <sup>11</sup>.

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<sup>7</sup>. Voltage-dependent calcium channels serve as crucial mediators of exocytotic release, including in neurotransmitters<sup>13,23,31</sup>. Therefore, it is possible that VD-induced nongenomic action modulates exocytotic release in osteoblasts.

In addition to mediation of VD-induced increase in  $\text{Ca}^{2+}$  by VDRs, it is also possible that VD activates voltage-dependent calcium channels directly. For example, it has been reported that 1,4-dihydropyridines BAYK 8644 activates L-type voltage-dependent calcium channels in cardiac cells<sup>4</sup>. 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  $[\text{Ca}^{2+}]_i$  involved PI3K-Akt pathways<sup>32</sup>. This suggests the need for further study on VD pathways.

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