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1α,25(OH)$_2$-vitamin D$_3$ membrane-initiated calcium signaling modulates exocytosis and cell survival

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Abstract

1α,25(OH)$_2$-vitamin D$_3$ (1,25D) is considered a bone anabolic hormone. 1,25D actions leading to bone formation involve gene transactivation, on one hand, and modulation of cytoplasmic signaling, on the other. In both cases, a functional vitamin D receptor (VDR) appears to be required. Here we study 1,25D-stimulated calcium signaling that initiates at the cell membrane and leads to exocytosis of bone materials and increased osteoblast survival. We found that rapid 1,25D-induction of exocytosis couples to cytoplasmic calcium increase in osteoblastic ROS 17/2.8 cells. In addition, we found that elevation of cytoplasmic calcium concentration is involved in 1,25D anti-apoptotic effects via Akt activation in ROS 17/2.8 cells and non-osteoblastic CV-1 cells. In both cases, 1,25D-stimulated elevation of intracellular calcium is due in part to activation of L-type Ca^{2+} channels. We conclude that 1,25D bone anabolic effects that involve increased intracellular Ca^{2+} concentration in osteoblasts can be explained at two levels. At the single-cell level, 1,25D promotes Ca^{2+}-dependent exocytotic activities. At the tissue level, 1,25D protects osteoblasts from apoptosis via a Ca^{2+}-dependent Akt pathway. Our studies contribute to the understanding of the molecular basis of bone diseases characterized by decreased bone formation and mineralization.

Keywords: Akt; Apoptosis; L-Ca channels; Rapid actions; Vitamin D$_3$

1. Introduction

The bone anabolic hormone 1α,25(OH)$_2$-vitamin D$_3$ (1,25D) increases bone matrix formation by binding to the nuclear vitamin D receptor (VDR), which is abundantly expressed in osteoblasts [1,2]. In addition, 1,25D exerts rapid, membrane-initiated actions that involve modulation of cytoplasmic signal transduction pathways [3]. Non-genomic actions that lead to bone formation include exocytosis of bone materials and regulation of cell survival [4]. While genomic mechanisms have been extensively studied, the signaling involved in membrane-initiated 1,25D actions remains partially understood.

Here we study 1,25D membrane-initiated calcium signaling in relation with bone anabolic functions in osteoblasts. Rapid 1,25D-promoted elevation of cytoplasmic Ca^{2+} concentration in osteoblasts has been known for decades [5,6]. However, its physiological significance remains to be determined. We recently demonstrated, for the first time, that nanomolar concentrations of 1,25D promote exocytosis in osteosarcoma ROS 17/2.8 cells within 1–3 min [7,8]. Calcium-dependent regulated exocytosis has been described in several cell systems in response to different stimuli [9]. It has been shown that 1,25D rapidly potentiates L-type Ca^{2+} channel activities at low depolarizing potentials in the same cell line [10]. Therefore, we investigated the hypothesis that 1,25D modulation of Ca^{2+} channel activities couples to exocytosis in bone cells, and thus contributes to bone formation.

In addition, changes in intracellular Ca^{2+} concentration have been implicated in cell death and survival. It has been recently shown that 1,25D exerts anti-apoptotic effects in osteoblasts [11,12]. To investigate the link between 1,25D-promoted changes in intracellular Ca^{2+}, regulation of apoptosis and bone anabolic functions, we studied the involvement of L-type Ca^{2+} channels in cell death and survival in osteoblasts and non-osteoblastic cells expressing different levels of the VDR protein.

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Our study contributes to the understanding of the physiological significance and underlying signaling pathways of rapid 1,25D actions that involve changes in intracellular calcium in osteoblasts. More specifically, our results provide new insights into mechanisms of bone formation in response to this steroid hormone.

2. Materials and methods

2.1. Chemicals

1,25D (Biomol Research Laboratories Inc., Plymouth, PA) was stored as a stock solution in pure ethanol at −20 °C, in the dark. Nifedipine, S(−)Bay K8644, ionomycin, etoposide, and staurosporine (STSP) were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Rat osteosarcoma ROS 17/2.8 cells (kindly provided by A.W. Norman, University of California-Riverside) were cultured in Ham's F-12 medium (Sigma) containing 5% fetal bovine serum (FBS, Sigma) and 5% Serum Plus (JRH Biosciences, Woodland, CA), with the addition of L-glutamine and antibiotics, and 1.1 mM $CaCl_2$, at 37 °C in a humidified 5% CO_2 atmosphere, as described previously [13]. CV-1 cells (kindly provided by A.W. Norman, University of California-Riverside) were cultured in DMEM medium (Sigma) supplemented with 10% FBS and antibiotics. Neuroblastoma-glioma hybridoma NG108-15 cells (a kind gift from M.E. Adams, University of California-Riverside) were cultured in DMEM medium supplemented with 1% HAT (hypoxanthine, aminopterin, and thymidine, Sigma), 10% FBS, L-glutamine and antibiotics.

Typically, cells were used 4–6 days after passage, at about 80% confluence. Culture medium was replaced by serum-free medium 24 h before treatment with 1,25D and/or the specified reagent. For apoptosis assays, cells were pretreated with 1,25D and/or specific reagent, or 0.01% ethanol for vehicle control, for an additional hour, and followed by 8 h exposure to 100 μM etoposide or 100 nM staurosporine (STSP) to induce apoptosis. For calcium measurements, 10 nM 1,25D or stimulating agent was added to the cells during the course of the experiments by means of automated injection in a multiplate reader.

2.3. Flow cytometry

Following apoptosis induction, cells were washed in ice-cold PBS buffer, trypsinized, centrifuged and resuspended in PBS buffer supplemented with 1% FBS. Cells were then fixed with methanol at 4 °C, overnight, and stained with 0.2 mg/ml μM propidium iodide (PI) at 37 °C for 2 h. Percentage of apoptotic cells (M1 fraction) was measured with a FACScan flow cytometer (Becton Dickinson, Becton Drive Franklin Lakes, NJ).

2.4. Calcium measurements

Changes in intracellular Ca^{2+} concentration were measured with the calcium sensitive dye Fluo-4 (Fluo-4 NW calcium assay kit, Molecular Probes) in a multi-detection microplate reader (Synergy HT, Bio-TECK Instruments, Winooski, VT) as described elsewhere [14]. Briefly, ROS 17/2.8 cells were seeded in black walled, clear-bottomed 96 well plates at a density of 15,000 cells/well. Cells were cultured for 3 days in Ham's F-12 medium as described before, and transferred to serum free medium 24 h before use. Cells were washed twice with Hank's Hepes buffer (HBSS) and loaded with 100 μl of Fluo-4 NW dye mix according to the manufacturer's protocol. Samples were excited at 494 nm and fluorescence emission was measured at 516 nm. Recordings were collected from each well, in a kinetic mode, for approximately 3 min.

2.5. Immunocytochemistry

Cells were cultured on cover slips in 35 mm Petri dishes for 48 h, fixed with 3.7% (v/v) formaldehyde (Sigma) at room temperature for 20 min, permeabilized with ice-cold ethanol for 5 min, and incubated with 5% goat serum at room temperature for 1 h to reduce background staining. Cells were incubated with a primary antibody against VDR (C-20, Santa Cruz Biotechnology, Delaware, CA) at 4 °C overnight, and a Cy3 anti-rabbit secondary antibody (Sigma Immunochemicals) (1:500 dilution) for 2 h at room temperature. Nuclei were stained with 300 nM 4′-6-diamino-2-phenylindole dihydrochloride (DAPI, Molecular Probes). Immunostaining was visualized in an inverted Olympus IX50 fluorescence microscope. Images were taken with a Spot Pursuit digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

2.6. Western blot analysis

Equal volumes of cell lysates were loaded and separated on 7.5–10% SDS-PAGE gels, and transferred to PVDF membranes. After blockade with 5% non-fat milk in TBST buffer, membranes were incubated with anti-phospho-Akt (ser 473) primary antibody (Cell Signaling Technology, Cummings Center, Beverly, MA), or an anti-VDR antibody (C-20, Santa Cruz Biotechnology). Primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody (Pierce, Meridian Rd., Rockford, IL) and enhanced chemiluminescence. Blot density was digitalized and analyzed by using Un-Scan-It gel software (Silk Scientific Inc., Orem, UT).

2.7. Electrophysiology

Whole-cell capacitance, a measure for exocytosis, was recorded with the patch-clamp technique on single osteoblasts essentially as described before [7].