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1α,25-Dihydroxyvitamin D_3 Rapidly Increases Nuclear Calcium Levels in Rat Osteosarcoma Cells

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Abstract 1α,25-Dihydroxyvitamin D_3 increases intracellular calcium in rat osteoblast-like cells that possess the classic receptor (ROS 17/2.8) as well as those that lack the classic receptor (ROS 24/1), indicating that a separate signalling system mediates this rapid nongenomic action. To determine the intracellular sites of this calcium increase, cytosolic and nuclear fluorescence (340 nm/380 nm ratio) were measured in Fura 2AM loaded ROS 17/2.8 cells using digital microscopy. Within 5 min, cytosolic fluorescence increased by 29% ($P < 0.05$) and nuclear fluorescence by 30% ($P < 0.01$) after exposure to 1α,25-dihydroxyvitamin D_3 (20 nM). This effect was blocked by the inactive epimer 1β,25-dihydroxyvitamin D_3. In an individual cell, cytosolic and nuclear fluorescence increased gradually after 1, 3, and 5 min exposure to vitamin D. Nuclei were then isolated from ROS 17/2.8 cells to directly measure the hormone's effect on nuclear calcium. The calcium content of Fura 2AM loaded nuclei was not affected by increasing the calcium concentration in the incubation buffer from 50 nM to 200 nM. After 5 min, 1α,25-dihydroxyvitamin D_3, 20 nM, increased the calcium of isolated nuclei in medium containing 50 nM calcium and 200 nM calcium. 1β,25-dihydroxyvitamin D_3, 20 nM, had no effect on nuclear calcium but blocked the 1α,25-dihydroxyvitamin D_3 induced rise in the isolated nuclei. The results indicate that the nuclear membrane of the ROS 17/2.8 cells contain calcium permeability barriers and transport systems that are sensitive to and specific for 1α,25-dihydroxyvitamin D_3.

1α,25-Dihydroxyvitamin D_3 rapidly increases nuclear calcium levels in both intact cells and isolated nuclei suggesting that rapid nongenomic activation of nuclear calcium may play a functional role in osteoblastic activity.

Key words: FURA 2 fluorescence, ROS 17/2.8 cells, steroid hormone, calcium, osteoblastic activity

The calcium ion, Ca^{+2}, plays an important role in the regulation of both intracellular enzymes and nuclear function. Ca^{+2} may serve as a signal to regulate gene expression [Vidair and Rubin, 1982], the growth of transformed cells [Tupper and Zorgmotti, 1977], expression of proto-oncogenes [Morgan and Curran, 1986, 1988], DNA fragmentation [Jones et al., 1989; McConkey et al., 1989; Nicotera et al., 1989], and nuclear enzymes [Jones et al., 1989].

The steroid hormone, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], participates in the regulation of plasma Ca^{+2}. In addition, the secosteroid rapidly and specifically increases intracellular Ca^{+2} in osteoblast-like cells [Baran et al., 1991; Civitelli et al., 1989; Lieberherr, 1987], and a variety of other cell types [Baran et al., 1990; Desai et al., 1986; Holick et al., 1987; Sugimoto et al., 1987]. Changes in intracellular Ca^{+2} modulate 1α,25-$(OH)_2D_3$-induced increases in osteocalcin mRNA transcription and steady-state levels in rat osteosarcoma cells ROS 17/2.8, suggesting that changes in intracellular Ca^{+2} may play a functional role in the hormone's effects on osteoblasts [Baran et al., 1992]. Extracellular Ca^{+2} and 1α,25-$(OH)_2D_3$ acting together can also modulate the expression of the calbindin gene in kidney [Clemens et al., 1989] and intestine [Brehier et al., 1989]. Since these studies did not measure intracellular calcium, it is not clear whether actual changes in cell calcium affect the action of vitamin D on calbindin expression in kidney and intestine.

Recent studies suggest that the nuclear membrane contains Ca^{+2} permeability barriers and transport systems that are hormonally sensitive [Waybil et al., 1991]. Vasopressin [Waybil et al.,

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1991], erythropoietin [Yelamarty et al., 1990], and histamine [Himpens et al., 1992] increase nuclear Ca^{+2} concentrations in target cells. The results of the present study indicate that 1α,25-$(OH)_2D_3$ increases nuclear Ca^{+2} levels in ROS 17/2.8 cells and the nuclear Ca^{+2} content of isolated nuclei within 5 min. These increments also occur in rat osteosarcoma cells, ROS 24/1, which lack the functional receptor [Baran et al., 1991] and in nuclei isolated from the ROS 24/1 cells indicating that the classic vitamin D receptor is not required for the rapid actions of 1α,25-$(OH)_2D_3$ on nuclear Ca^{+2}.

METHODS

Cell Cultures

Osteoblast-like rat osteosarcoma cells, ROS 17/2.8 (generously supplied by Dr. G. Rodan, Merck Sharp Dohme, West Point, PA) and ROS 24/1 (generously supplied by Dr. M. Haussler, University of Arizona) were grown in culture medium consisting of DMEM:F12 (50:50) plus 10% fetal calf serum.

Cells were grown for 6–7 days and harvested for experiments by trypsinization with 0.25% trypsin and 0.002% EDTA and by sedimentation at 100*g* for 8 min. Cells are only studied up to facility passage 10. Cell numbers were assessed by counting an aliquot of cells in a hemocytometer, and viability was determined by trypan blue dye exclusion.

Determination of Intracellular Calcium Levels by Fura 2 Fluorescence

Intracellular Ca^{+2} was measured in ROS 17/2.8 and ROS 24/1 cells in a cuvette as previously described [Baran et al., 1991]. Fura 2AM (Molecular Probes, Eugene, OR), 100 nmoles, in 10 μl of dimethylsulfoxide (DMSO), was added to 10^8 cells in 10 ml of HEPES-BSS with albumin for 60 min and centrifuged at 50*g*. Cells were resuspended in 10 ml of HEPES-BSS (without albumin) and treated with 1α,25-$(OH)_2D_3$, 20 nM, or its inactive epimer in the presence and absence of 1α,25-$(OH)_2D_3$ and fluorescence measured after 5 min.

The 1α,25-$(OH)_2D_3$ was provided courtesy of Dr. M. Uskovic, Hoffmann-LaRoche, Inc., Nutley, NJ while 1β,25-$(OH)_2D_3$ was provided courtesy of Dr. M. Holick of Boston University.

Measurement of Fura 2 Fluorescence in Single Cells

Rat osteosarcoma cells ROS 17/2.8 were loaded with Fura 2 AM as previously described. The cells were diluted and individual cells were observed using the digital imaging microscope [Moore et al., 1990] and a Nikon 40× UVF (NA1 1.3) objective. Images of Fura 2 fluorescence at 500 nm emission were obtained with 340 nm and 380 nm excitation wavelengths. The total fluorescence intensity emitted from the cytoplasm and nucleus of individual cells was determined using interactive computer graphics software to outline the cell and the nucleus. These regions were easily distinguished from one another by the step change in fluorescence intensity at the nuclear-cytoplasm interface. The ratio of fluorescence intensity at 340/380 excitation wave lengths was calculated to obtain a measure of Ca^{+2} changes in response to various treatments. The cells were treated on the coverslip with 1α,25-$(OH)_2D_3$ and observed over time. Images were obtained at 1, 3, and 5 min to determine the significance of the observed changes in cytosolic and nuclear region fluorescence. The 340/380 fluorescence ratios were determined in five cells after exposure to ethanol (vehicle) 1α,25-$(OH)_2D_3$, 20 nM, 1β,25-$(OH)_2D_3$, 20 nM, or both. All cells in the field appeared similar and five representative cells were chosen at random.

Isolation of Nuclei by Hypotonic Swelling

Rat osteosarcoma cells, ROS 17/2.8 and ROS 24/1, were washed in an isotonic buffer consisting of 125 mM KCl, 30 mM Tris/HCl (pH 7.9), 5 mM $MgCl_2$, and 10 mM Beta Mercaptoethanol (BME). The cells were centrifuged and resuspended in 1 ml per 10^7 cells of ice cold Hypotonic Swelling Buffer [10 mM KCl, 30 mM Tris/HCl (pH 7.9), 5 mM $MgCl_2$, and 10 mM BME]. The cells were allowed to swell for 20 min on ice and then dounce homogenized with a tight fitting pestle for 15 strokes. This produced approximately 95% recovery of intact nuclei [Baran et al., 1992; Lian et al., 1989].

Determination of Calcium Levels in Isolated Nuclei

By the method of Nicotera [Nicotera et al., 1989], nuclei (10^7/ml) were loaded with 1 μM Fura 2 AM (Molecular Probes, Eugene, OR) for 60 min at 4°C. Nuclei were washed, resus-

pended, and equilibrated at 37°C under an atmosphere of 95% O_2:5% CO_2 for 15 min [Baran et al., 1989]. Nuclear Ca^{+2} was calculated using the 340/380 fluorescence ratio of Fura 2 loaded nuclei as indicated above.

Statistics

Values represent the mean ± SD. Statistical significance was determined by Student's *t* test or Duncan's test for multiple comparisons where appropriate.

RESULTS

As previously reported, 1α,25-$(OH)_2D_3$, 20 nM, increased intracellular Ca^{+2} levels in ROS 17/2.8 (112 ± 22 vs. 142 ± 34 nM, $P < 0.05$) and ROS 24/1 (91 ± 15 vs. 135 ± 14 nM, $P < 0.05$) cells after 5 min. To determine the intracellular sites of this increased calcium, cytosolic and nuclear fluorescence was measured in a population of ROS 17/2.8 cells using digital microscopy. After 5 min, 1α,25-$(OH)_2D_3$ increased the 340/380 fluorescence ratio in the cytoplasm (1.71 ± 0.41 vs. 2.20 ± 0.32, $P < 0.05$, n = 5) and nuclear region (1.95 ± 0.27 vs. 2.53 ± 0.24, $P < 0.01$, n = 5). This increase in both cytosolic and nuclear region fluorescence with 1α,25-$(OH)_2D_3$ was blocked by pretreatment with 1β,25-$(OH)_2D_3$ (1.73 ± 0.09 = cytoplasm, 1.99 ± 0.10 = nucleus). An individual ROS 17/2.8 cell showed increased cytosolic (1.70 vs. 1.96 vs. 2.01) and nuclear region (1.71 vs. 1.97 vs. 2.20) fluorescence (Fig. 1) when measured after 1, 3, and 5 min exposure to 1α,25-$(OH)_2D_3$.

Nuclei were isolated from ROS 17/2.8 cells to directly measure the hormone's effect on nuclear calcium. The calcium content of isolated nuclei from ROS 17/2.8 cells was not affected by the calcium content of the Fura 2 loading and incubation buffer. Increasing calcium concentration from 50 nM to 200 nM had no effect on intranuclear Ca^{+2} (161 ± 54 vs. 135 ± 27 nM). In medium containing 200 nM Ca^{+2}, 1α,25-$(OH)_2D_3$, 20 nM, increased the Ca^{+2} in nuclei isolated from ROS 17/2.8 cells within 5 min (135 ± 23 vs. 247 ± 33 nM, $P < 0.01$). In medium containing 50 nM Ca^{+2}, 1α,25-$(OH)_2D_3$ increased the Ca^{+2} in isolated nuclei from ROS 17/2.8 cells (204 ± 8 vs. 271 ± 46 nM, $P < 0.01$) within 5 min, while 1β,25-$(OH)_2D_3$, 20 nM, the biologically inactive epimer had no effect on nuclear Ca^{+2} (182 ± 17 nM) but blocked the 1α,25-$(OH)_2D_3$-stimulated increase (172 ± 20 nM) (Fig. 2). The 1α,25-$(OH)_2D_3$ induced increase in nuclear Ca^{+2} is not a result of binding to the classic vitamin D receptor since the hormone also increased Ca^{+2} within 5 min in nuclei isolated from ROS 24/1 cells, which lack the classic nuclear vitamin D receptor (142 ± 78 vs. 242 ± 26 nM, $P < 0.05$).

DISCUSSION

1α,25-$(OH)_2D_3$ rapidly and specifically increases intracellular Ca^{+2} in ROS 17/2.8 cells and in ROS 24/1 cells which lack the classical vitamin D receptor [Baran et al., 1991]. This hormone-induced increment in Ca^{+2} can be blocked by 1β,25-$(OH)_2D_3$, the inactive epimer of vitamin D [Baran et al, 1991]. 1β, 25 $(OH)_2D_3$ does not bind to nor displace 1α,25-$(OH)_2D_3$ from the classical receptor [Holick et al., 1980]. These findings indicate that the 1α,25-$(OH)_2D_3$-induced increases in intracellular calcium are mediated by a vitamin D signalling system different from the classical mechanism. These changes in intracellular Ca^{+2} appear to have functional significance in regulating 1α,25-$(OH)_2D_3$ induced increments in osteocalcin mRNA transcription and steady-state levels [Baran et al., 1992].

In this study using digital microscopy, we have examined the changes in cytosolic and nuclear region fluorescence of Fura 2 AM loaded ROS 17/2.8 cells after exposure to vitamin D. Changes in cytosolic and nuclear region fluorescence after 3 and 5 min of 1α,25-$(OH)_2D_3$ treatment were demonstrated in a single cell (Fig. 1). These increments were determined by measuring the 340/380 fluorescence ratio in the cytosol and nuclear region of five cells after a 5 min exposure to 1α,25-$(OH)_2D_3$, 20 nM, or vehicle and demonstrated that 1α,25-$(OH)_2D_3$ increased nuclear region Ca^{+2} levels within the ROS cells. Given the three-dimensional nature of the cell, the nuclear region Ca^{+2} levels measured must be interpreted as the summation of effects occurring in the cytosol above and below the nucleus as well as within the nucleus. These observations are consistent with the findings that nuclear Ca^{+2} gradients can be maintained in hepatocytes, smooth muscle cells, erythroblasts, neutrophils, and lymphocytes [Waybil et al., 1991; Himpens et al., 1992; Williams et al., 1985; Yelamarty et al., 1990]. Waybil et al. [1991] conclude that the hepatic nuclear membrane contains Ca^{+2} permeability barriers and Ca^{+2}

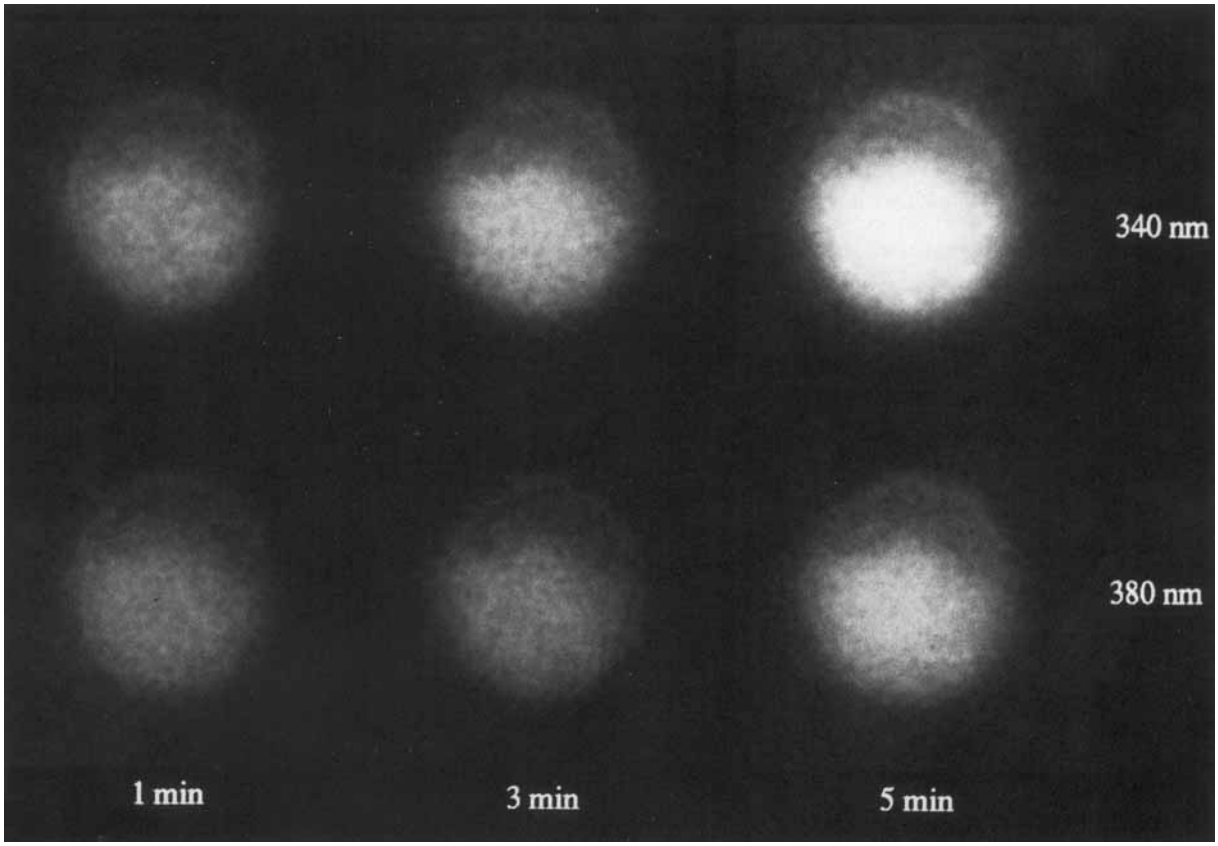

Fig. 1. Fluorescence images in a single ROS 17/2 8 cell loaded with Fura 2 following exposure to $1\alpha,25\text{-}(OH)_2D_3$ Images at fluorescence exciting at 340 and 380 are shown at 1, 3, and 5 min after $1\alpha,25\text{-}(OH)_2D_3$ treatment The 340/380 ratio for the nucleus is 1 71, 1 97, and 2 20 at 1, 3, and 5 min, respectively

transport mechanisms that are hormonally sensitive and that increased nuclear Ca^{+2} may regulate cell proliferation by activating Ca^{+2}-dependent endonucleases, calmodulin, or protein kinase C (PKC). Similarly, erythropoietin has been shown to increase the nuclear Ca^{+2} concentration of erythroblasts by 200–300% [Yelamarty et al., 1990], and histamine has been shown to increase the nuclear Ca^{+2} of smooth muscle cells [Himpens et al., 1992].

We have previously shown that $1\alpha,25\text{-}(OH)_2D_3$ rapidly increased intracellular Ca^{+2} in rat osteoblast-like cells by release from intracellular stores [Baran et al., 1991]. In the present study, we demonstrate that nuclear Ca^{+2} levels are maintained when the nuclei are resuspended in 50 nM Ca^{+2} or 200 nM Ca^{+2} buffers. Increased cytosolic Ca^{+2} has been reported to have no effect on nuclear Ca^{+2} in smooth muscle cells, indicating that the nuclear Ca^{+2} compartment is regulated independently of the cytoplasm's compartment by nuclear membrane dependent processes [Williams et al., 1985]. Recently, Himpens et al. [1992] showed a difference in regulation between nuclear and cytoplasmic Ca^{+2} in cultured smooth muscle cells during agonist stimulation. It has also been demonstrated that Ca^{+2} within the nucleus can be released from intranuclear sites by PKC in Swiss 3T3 cells [Martelli et al., 1990]. Although the primary location and activation of PKC is thought to be the plasma membrane, recent reports indicate that there is a translocation of PKC to the nucleus and thus intranuclear targets for PKC [Divecha et al., 1991].

In addition, the presence of an entirely separate nuclear phosphoinsitide signalling system has been suggested by the observation that purified nuclei can synthesize polyphosphoinositide lipis in vitro [Divecha et al., 1991]. Inositol trisphosphate, produced in response to $1\alpha,25\text{-}(OH)_2D_3$ in rat osteosarcoma cells [Civitelli et al., 1990] stimulates PKC dependent protein phosphorylation in isolated nuclei [Martelli et al., 1990]. Thus, $1\alpha,25\text{-}(OH)_2D_3$-induced increases in nuclear Ca^{+2} within the cell may be

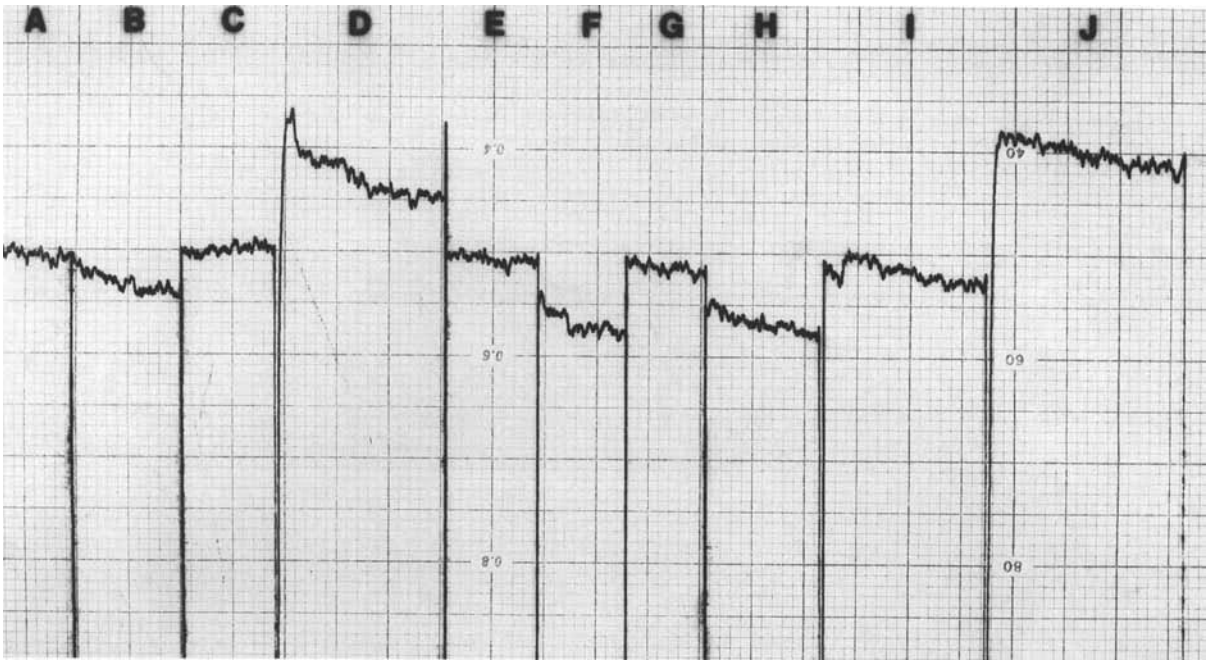

Fig. 2. Fluorescence of Fura 2 loaded nuclei from ROS 17/2 8 cells Fluorescence at 340 nm of (A) no treatment vs (B) alcohol, (C) no treatment vs (D) $1\alpha,25\text{-}(OH)_2D_3$ (20 nM), (E) no treatment vs (F) $1\beta,25\text{-}(OH)_2D_3$ (20 nM), (G) no treatment vs (H) $1\beta,25\text{-}(OH)_2D_3$ (20 nM), added 30 secs prior to $1\alpha,25\text{-}(OH)_2D_3$ (20 nM), and (I) no treatment vs (J) $1\alpha,25\text{-}(OH)_2D_3$ (20 nM)

mediated by translocation of PKC within the cell, or activation of a nuclear phospholipid signalling system [Baran et al., 1989].

$1\beta,25\text{-}(OH)_2D_3$, the vitamin D epimer which does not interact with the classic receptor, inhibits the rapid effects of $1\alpha,25\text{-}(OH)_2D_3$ in hepatocytes [Baran et al., 1990], isolated hepatic nuclei [Baran et al., 1989], ROS cells [Baran et al., 1991], and in isolated ROS 17/2.8 nuclei (Fig. 2), suggesting that the epimer binds to but does not activate the signalling system mediating the rapid actions at the nuclear level. Structure function studies using vitamin D analogs suggest that there may exist distinctive forms of the $1\alpha,25\text{-}(OH)_2D_3$ receptor that are involved in genomic and nongenomic activation [Farach-Carson et al., 1991].

We have previously shown that the inactive $1\beta,25\text{-}(OH)_2D_3$ inhibits the $1\alpha,25\text{-}(OH)_2D_3$-induced increases in osteoblast calcium, osteocalcin mRNA transcription, and osteocalcin mRNA steady-state levels without affecting the sequence specific binding of the vitamin D receptor complex to the VDRE of the OC gene. Thus, the rapid effects of $1\alpha,25\text{-}(OH)_2D_3$ appear to be mediated by a separate signalling system. Inhibition of the rapid effects blocks the genomic actions of the hormone on OC expression, perhaps by affecting the hormone's transactivation function [Baran et al., 1992]. Therefore, the net effect of $1\alpha,25\text{-}(OH)_2D_3$ on cell function may reflect the combined genomic and non-genomic actions of the hormone.

We conclude that the osteoblastic nuclear membrane contains Ca^{+2} permeability barriers and Ca^{+2} transport mechanisms which are sensitive to the seco-steroid $1\alpha,25\text{-}(OH)_2D_3$. $1\alpha,25\text{-}(OH)_2D_3$ specifically and rapidly increases nuclear Ca^{+2} levels in ROS cells perhaps by influx from perinuclear stores and/or by release of intranuclear Ca^{+2}. This rapid and nongenomic vitamin D mediated activation of Ca^{+2} may play a functional role in osteoblastic activity.

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