Journal of Cellular Biochemistry 53:234–239 (1993)

1α,25-Dihydroxyvitamin D_3-Induced Changes in Intracellular pH in Osteoblast-Like Cells Modulate Gene Expression

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Abstract 1α,25-Dihydroxyvitamin D_3 exerts rapid nongenomic effects on rat osteoblast-like cells independent of the classic nuclear receptor. These effects include changes in phospholipid metabolism and cell calcium. Intracellular calcium itself has been proposed to regulate intracellular pH in osteoblast cell lines. The purpose of this study was to determine the effect of 1α,25-dihydroxyvitamin D_3 on intracellular pH, the relationship of changes in calcium to changes in pH, and the role of pH changes in genomic activation. 1α,25-Dihydroxyvitamin D_3 increased intracellular pH within 10 min in rat osteoblast-like cells, an effect that was inhibited by removal of extracellular sodium and by the biologically inactive epimer 1β,25-dihydroxyvitamin D_3. The hormone increased intracellular calcium in Quin 2 loaded cells in the presence and absence of extracellular sodium. The 1α,25-dihydroxyvitamin D_3-induced increments in osteocalcin and osteopontin mRNA levels were abolished in sodium-free medium. The results indicate that 1α,25-dihydroxyvitamin D_3-induced increments in cellular calcium precede cell alkalinization and that these changes in intracellular pH may modulate steady-state mRNA levels of genes induced by vitamin D.

Key words: 1,25-Dihydroxyvitamin D_3 osteoblasts, intracellular pH, gene expression, mRNA, cell calcium

1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) is known to modulate gene expression [Minghetti and Norman 1988]. Recently, the hormone has been shown to exert rapid nongenomic effects in osteoblasts. These nongenomic effects include changes in intracellular calcium [Lieberherr, 1987; Baran et al., 1991], nuclear calcium [Sorensen et al., 1993], and phospholipid metabolism [Civitelli et al., 1990] and appear to modulate the genomic actions of the hormone [Baran et al., 1992]. Moreover, 1α,25-$(OH)_2D_3$ exerts rapid effects in osteoblast-like cells that lack the classic nuclear vitamin D receptor indicating that a separate signaling system mediates the rapid effects [Baran et al., 1991].

Intracellular calcium has been shown to be involved in the regulation of intracellular pH in osteoblastic cells [Green and Kleeman, 1992]. Cell alkalinization is thought to be necessary for the initiation of DNA synthesis [Pouyssegur et al., 1984]. When cytosolic alkalinization is inhibited, the mitogenic effect of growth factors is lost [Pouyssegur et al., 1984; Paris, Pouyssegur, 1984], and it is suggested that the rise in cytosolic pH may serve as a signal for mitogenesis [Thomas, 1989, see Ref. 11 for review]. The osteoblast possesses receptors for both parathyroid hormone (PTH) and 1α,25-$(OH)_2D_3$ and both hormones increase intracellular calcium in osteoblasts [Lieberherr, 1987; Baran et al., 1991; Sorensen et al., 1993; Baran et al., 1992; Donahue et al., 1988; Donahue et al., 1990; Yamada et al., 1989; Van Leeuwen et al., 1988; Yamaguchi et al., 1987]. However, whereas PTH decreases intracellular pH in osteoblast-like cells [Reid et al., 1988], 1α,25-$(OH)_2D_3$ has been shown to increase intracellular pH in hepatocytes [Baran and Kelly, 1988] and monocytic-like cells [Hill et al., 1989]. The effect of 1α,25-$(OH)_2D_3$ on intracellular pH and the relationship of intracellular pH changes to vitamin D-induced alterations in gene expression in osteoblasts is unknown.

The results of this study demonstrate that (1) 1α,25-$(OH)_2D_3$ increases intracellular pH in osteoblasts, (2) this effect is abolished in the absence of extracellular sodium and is the result of

Received July 23, 1993; accepted July 26, 1993.

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activation of the same signaling system that mediates the vitamin D-induced rapid changes in intracellular calcium, and (3) intracellular alkalinization plays a role in $1\alpha,25\text{-}(OH)_2D_3$ induction of osteocalcin and osteopontin gene expression in the osteoblast.

MATERIALS AND METHODS

Cell Cultures

Rat osteosarcoma cells (ROS 17/2.8) were grown and maintained in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) and F12 (50:50) plus 5% fetal calf serum. Cells were grown for 6–7 days and harvested for experimentation by trypsinization with 0.25% trypsin and 0.002% EDTA followed by sedimentation at 200 *g* for 8 min. 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 Quin 2 Fluorescence

Cytosolic calcium was measured as previously described [Baran et al., 1991]. The ROS cells, 1×10^8, were incubated for 15 mins in 10 ml HEPES–BSS (buffered salt solution) containing 0.15 g albumin at 37°C under O_2 and CO_2 (95:5). Quin-2AM (CalBiochem-Behring Corp., San Diego, CA), 1,000 nmol, in 10 μl dimethylsulfoxide (DMSO), was added to 10 ml cells for 30 minutes. The cells were diluted to 15 ml with the HEPES–BSS (without albumin) and centrifuged at 50 *g*. These were resuspended in 10 ml HEPES–BSS (without albumin) and incubated for an additional 15 mins.

Cellular fluorescence was determined in a 650-10S scanning fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Fluorescence reading (F) were taken at an emission wavelength of 495 nm and a slit width of 5 nm and an excitation wavelength of 339 nm and a slit width of 5 nm with constant stirring (Spectrocell, Inc., Oreland, PA). The emission spectrum was determined by scanning between emissions of 380 and 530 nm. Recordings were made on an R100A recorder (Perkin-Elmer Corp.). Maximal fluorescence was determined by the addition of 100 μl 10% Triton X-100 to lyse 1-ml cells (F_{max}) and minimal fluorescence by the addition of 50 μl of 1 M EGTA to chelate the calcium (F_{min}). The pH of the lysed cells after addition of EGTA was 8.50. Corrections were made for fluorescence in DMSO-treated cells, the quenching of fluorescence due to Triton X-100, and the effects of any solvent, such as alcohol. The cytosolic calcium content was determined by the equation

$$[Ca^{2+}]_i = K_D(F - F_{min})(F_{max} - F)$$

where the dissociation constant K_d is 115 nM. When indicated, $1\alpha,25\text{-}(OH)_2D_3$ (courtesy Dr. M. Uskovic, Hoffmann-LaRoche Inc., Nutely, NJ) or $1\beta,25\text{-}(OH)_2D_3$ (courtesy Dr. M. Holick of Boston University) was added to 1 ml cells in 10 μl of 95% alcohol for 5 mins. Controls were treated with 10 μl of 95% alcohol for 5 mins.

Determination of Intracellular pH by Biscarboxyethyl Carboxyfluorescein (BCECF) Fluorescence

The ROS cells (1×10^8 in 10 ml HEPES–BSS with albumin) were incubated with BCECF (Molecular Probes; 0.1 μM) for 30 min. The cells were then centrifuged at 50 *g* for 2 min and resuspended in albumin-free buffer for a 5-min equilibration period. One-ml aliquots of the cell suspension were treated with $1\alpha,25\text{-}(OH)_2D_3$, $1\beta,25\text{-}(OH)_2D_3$, or vehicle.

Cellular fluorescence was determined in a Perkin-Elmer 650-10S scanning fluorescence spectrophotometer. Fluorescence readings were recorded at an excitation of 500 nm and a slit width of 3 nm, and an emission of 530 nm and a slit width of 4 nm. After fluorescence readings were recorded, these same aliquots were used to generate a standard curve by lysing the cells with Triton and measuring the fluorescence signal of the released dye at known pH [Rink et al., 1982].

To determine the effect of extracellular sodium on intracellular pH and intracellular calcium, cells were suspended in HEPES-BSS, containing choline substituted for sodium to maintain osmolality, for 5 min prior to exposure to $1\alpha,25\text{-}(OH)_2D_3$.

Steady-State mRNA Levels

Total cellular RNA was isolated from the ROS 17/2.8 cells exposed to $1\alpha,25\text{-}(OH)_2D_3$, 20 nM, and or sodium-free medium for 3 hr by the commercial RNAzol method (Cinna/Biotex Labs, Friendswood, TX). Integrity of the RNA preparations was determined by ethidium bromide staining of electrophoretically fractionated total cellular RNA; a 2:1 ratio of 28s to 18s ribosomal RNAs was established by microdensitometry.

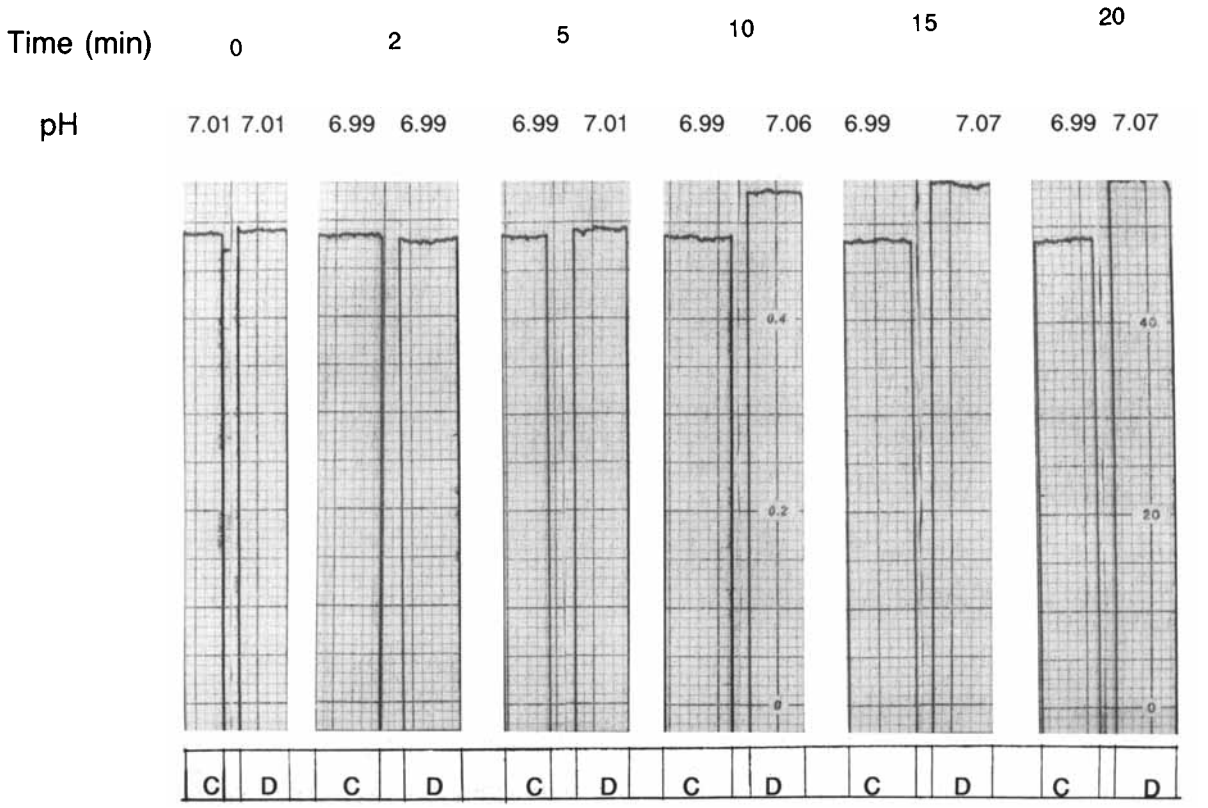

Fig. 1. Time course of $1\alpha,25\text{-}(OH)_2D_3$-induced changes in intracellular pH in ROS 17/2.8 cells. Fluorescence was assessed in two 1 ml aliguots of cells treated with $1\alpha,25\text{-}(OH)_2D_3$ or alcohol for the designated times. This same cell preparation was then used to generate the standard curve as described in Methods.

Osteocalcin, osteopontin, and alkaline phosphatase mRNA levels were analyzed by slot blot. The RNA was hybridized to ^{32}P-radiolabeled rat genomic clones osteocalcin, pOC 34. [Lian, et al., 1989], osteopontin [Oldberg et al., 1986], or alkaline phosphatase (pRAP54) [Noda et al., 1987]. The uniformity of RNA application, 10 μg, was confirmed by quantitation of ribosomal RNA hybridized with a ^{32}p-labeled 28S ribosomal RNA genomic clone (LS-6) [Wilson et al., 1978].

Statistics

Probability of difference was determined by the paired *t*-test for the rapid effects and the results represent the mean ± SD. Changes in steady-state mRNA levels were determined by *Duncan's* test for multiple comparisons.

RESULTS

Cell viability was greater than 95% in the presence and absence of extracellular sodium. $1\alpha,25\text{-}(OH)_2D_3$ had no effect on cell viability.

As previously reported [Baran et al., 1991], $1\alpha,25\text{-}(OH)_2D_3$, 20 nM, increased intracellular calcium in ROS 17/2.8 cells incubated in sodium containing buffer (160 ± 2 vs. 244 ± 15 nM, $P < 0.001$, n = 5). Substitution of choline for sodium did not block the intracellular calcium response to $1\alpha,25\text{-}(OH)_2D_3$ (160 ± 24 vs. 241 ± 33 nM, $P < 0.05$, n = 5).

$1\alpha,25\text{-}(OH)_2D_3$, 20 nM, gradually increased BCECF fluorescence in ROS 17/2.8 cells (Fig. 1). At 10 min, the hormone had significantly increased intracellular pH (7.18 ± 0.02 vs. 7.26 ± 0.02, $P < 0.05$, n = 4). $1\alpha,25\text{-}(OH)_2D_3$, 200 nM, did not further increase intracellular pH (7.25 ± 0.01, n = 5). $1\beta,25\text{-}(OH)_2D_3$, 20 nM, had no effect on intracellular pH (7.17 ± 0.03) but when added 30 sec prior to $1\alpha,25\text{-}(OH)_2D_3$, 20 nM, blocked the vitamin D induced intracellular alkalinization (7.19 ± 0.04, n = 5). Likewise, removal of extracellular sodium and substitution with choline blocked the $1\alpha,25\text{-}(OH)_2D_3$ effect on intracellular pH (7.11 ± 0.04 vs. 7.13 ± 0.02, n = 4).

Steady-state osteocalcin and osteopontin mRNA levels increased after 3 hr in the presence of $1\alpha,25\text{-}(OH)_2D_3$ and sodium (Table I; Fig.

TABLE I. Steady-State mRNA Values of Osteocalcin, Osteopontin, and Alkaline Phosphatase in ROS 17/2.8 Cells After Exposure to 1α, 25-$(OH)_2D_3$ and/or Removal of Extracellular Sodium†

	Control	1α,25-$(OH)_2D_3$	Choline control	Choline + 1α,25-$(OH)_2D_3$
Osteocalcin	0.80 ± 0.46	5.03 ± 1.78*	0.68 ± 0.38	0.64 ± 0.45
Osteopontin	0.24 ± 0.06	0.33 ± 0.03*	0.19 ± 0.04	0.17 ± 0.05
Alkaline Phosphatase	0.23 ± 0.04	0.15 ± 0.05	0.22 ± 0.09	0.14 ± 0.05

†Values represent the mean ±SD of four observations in each group and reflect mRNA levels in densitometry units normalized to the LS6 ribosomal RNA.
*$P > 0.05$ compared to other groups as determined by Duncan's test for multiple comparisons.

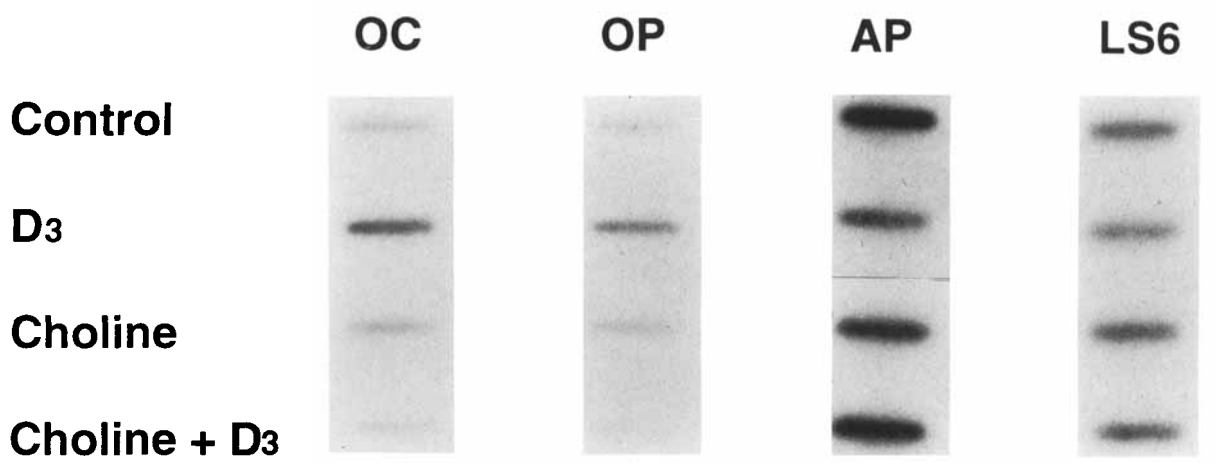

Fig. 2. Hybridization analysis. The effect of 1α,25-$(OH)_2D_3$ on osteocalcin (OC), osteopontin (OP), alkaline phosphatase (AP) and ribosomal (LS6) mRNA in the presence and absence of extracellular sodium in ROS 17/2.8 cells.

2). Removal of extracellular sodium had no significant effect on basal expression of either osteocalcin or osteopontin, but inhibited the 1α,25-$(OH)_2D_3$-induced increase in osteocalcin and osteopontin mRNA levels (Table I; Fig. 2). Steady-state levels of alkaline phosphatase mRNA were unaffected by three hours exposure to 1α,25-$(OH)_2D_3$ or removal of extracellular sodium.

DISCUSSION

The results indicate that 1α,25-$(OH)_2D_3$ induces rapid intracellular alkalinization (within 10 min) in osteoblast-like cells, which appears to be regulated by the Na^+/H^+ antiport. Removal of extracellular sodium and substitution with choline blocked the 1α,25-$(OH)_2D_3$-mediated increments in osteocalcin and osteopontin mRNA steady-state levels without affecting cell viability. This indicates that vitamin D-induced cell alkalinization may be functionally important for gene expression. Removal of extracellular sodium does not block the vitamin D-induced rapid increase in intracellular calcium indicating that the rapid changes in cell calcium precede alkalinization. Previous studies have demonstrated that the rapid vitamin D-induced changes in intracellular calcium occur in osteoblast-like cells that lack the mRNA for the vitamin D receptor [Baran et al., 1991]. Furthermore, 1β,25-$(OH)_2D_3$ inhibits the rapid effects of 1α,25-$(OH)_2D_3$ in osteoblast-like cells that both lack (ROS 24/1) and possess (ROS 17/2.8) the classical nuclear vitamin D receptor. The 1β,25-$(OH)_2D_3$-induced inhibition occurs in cells that possess the nuclear vitamin D receptor without altering the binding of the 1α,25-$(OH)_2D_3$-receptor complex to DNA [Baran et al., 1992]. Thus, the rapid effects of 1α,25-$(OH)_2D_3$ are mediated by a signalling system separate from the classic nuclear receptor, and sensitive to inhibition by 1β,25-$(OH)_2D_3$. Since 1β,25-$(OH)_2D_3$ inhibits both the 1α,25-$(OH)_2D_3$-induced rapid changes in cell calcium and pH, both of these rapid effects appear to be the result of activation of the same signaling system.

Na^+/H^+ exchange plays a major role in the regulation of intracellular pH in osteoblasts. In rat calvarial osteoblasts, inhibition of the antiport system by removal of extracellular sodium or addition of amiloride blocks recovery from an acid load [Redhead, 1988]. It has been suggested that osteoblast regulation of intracellular pH and pH in the microenvironment may play a role in bone formation [Green et al., 1990]. Crosslinking of collagen and its subsequent mineralization are pH-dependent processes requiring extracellular alkalinization. Transport of protons by osteoblasts may modify the extracellular pH thereby influencing bone formation [Green et al., 1988, 1990, 1992].

It is well established that osteoblast-produced factors influence osteoclast activity. Other studies have demonstrated the importance of Na^+/H^+ exchange in osteoclast function. Amiloride, a Na^+/H^+ antiport inhibitor, prevents bone resorption in vitro [Hall and Chambers, 1990]. The Na^+/H^+ antiport was found to be the primary mechanism to externalize H^+ during bone resorption suggesting the physiological importance of Na^+/H^+ exchange in osteoclast function [Hall and Chambers, 1990].

The interrelationships between intracellular pH and genomic activation remain unclear. Parathyroid hormone has been shown to inhibit the Na^+/H^+ antiport and ^3H-thymidine incorporation into DNA in UMR-106 cells suggesting a regulatory role for intracellular pH in DNA synthesis in these osteoblast-like cells [Reid et al., 1988]. In the present study it has been shown that inhibition of $1\alpha,25\text{-}(OH)_2D_3$-induced cell alkalinization in osteoblast-like cells alters the hormone induction of bone matrix protein genes. A previous report has demonstrated that inhibition of $1\alpha,25\text{-}(OH)_2D_3$-induced increments in intracellular Ca^{+2} prevents the hormone mediated increases in osteocalcin mRNA transcription [Baran et al., 1992]. In the absence of extracellular Na^+, $1\alpha,25\text{-}(OH)_2D_3$ induces a rapid increase in intracellular calcium and also does not increase steady-state mRNA levels of osteocalcin or osteopontin suggesting that cell pH may play an important role in gene transcription. It is not known whether the failure of vitamin D to increase steady-state mRNA levels in the absence of cellular alkalinization is due to diminished gene transcription, alteration of binding of the vitamin D–receptor complex to DNA, changes in other transcription factors, or altered stability of the mRNA transcripts. However, it appears that activation of the Na^+/H^+ antiport and subsequent cell alkalinization is required for genomic activation by $1\alpha,25\text{-}(OH)_2D_3$ in osteoblast-like cells.

The data presented in this report show the importance of intracellular alkalinization on vitamin D induction of osteocalcin and osteopontin genes and further support the hypothesis that the rapid actions of $1\alpha,25\text{-}(OH)_2D_3$ modulate the genomic effects of the hormone.

This work was supported by NIH grants AR 33920, AR39588, GM32010, and DK39085.

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