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Effects of the Vitamin D_3 Analog 1α,25-Dihydroxyvitamin D_3-3β-Bromoacetate on Rat Osteosarcoma Cells: Comparison With 1α,25-Dihydroxyvitamin D_3

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Abstract The actions of the hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], are mediated by both genomic and nongenomic mechanisms. Several vitamin D synthetic analogs have been developed in order to identify and characterize the site(s) of action of 1α,25-$(OH)_2D_3$ in many cell types including osteoblastic cells. We have compared the effects of 1α,25-$(OH)_2D_3$ and a novel 1α,25-$(OH)_2D_3$ bromoester analog (1,25-$(OH)_2$-BE) that covalently binds to vitamin D receptors. Rat osteosarcoma cells that possess (ROS 17/2.8) or lack (ROS 24/1) the classic intracellular vitamin D receptor were studied to investigate genomic and nongenomic actions. In ROS 17/2.8 cells plated at low density, the two vitamin D compounds (1×10^{-8} M) caused increased cell proliferation, as assessed by DNA synthesis and total cell counts. Northern blot analysis revealed that the mitogenic effect of both agents was accompanied by an increase in steady-state osteocalcin mRNA levels, but neither agent altered alkaline phosphatase mRNA levels in ROS 17/2.8 cells. ROS 17/2.8 cells responded to 1,25-$(OH)_2$-BE but not the natural ligand with a significant increase in osteocalcin secretion after 72, 96, 120, and 144 hr of treatment. Treatment of ROS 17/2.8 cells with the bromoester analog also resulted in a significant decrease in alkaline phosphatase–specific activity. To compare the nongenomic effects of 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE, intracellular calcium was measured in ROS 24/1 cells loaded with the fluorescent calcium indicator Quin 2. At 2×10^{-8} M, both 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE increased intracellular calcium within 5 min. Both the genomic and nongenomic actions of 1,25-$(OH)_2$-BE are similar to those of 1α,25-$(OH)_2D_3$, and since 1,25-$(OH)_2$-BE has more potent effects on osteoblast function than the naturally occurring ligand due to more stable binding, this novel vitamin D analog may be useful in elucidating the structure and function of cellular vitamin D receptors.

Key words: 1α,25-dihydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3-3β-bromoacetate, ROS 17/2.8, ROS 24/1, DNA synthesis, osteocalcin production, alkaline phosphatase activity, intracellular calcium

1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) exerts both endocrine and paracrine/autocrine effects on many cell types within bone [Stern, 1990]. Assessment of the effects of 1α,25-$(OH)_2D_3$ on parameters of osteoblast cell proliferation and differentiation in cultured normal and transformed osteoblasts has led to conflicting reports [Chen et al., 1983; Ishida et al., 1993]. The variable cell responses most likely reflect differences in cell culture systems, as well as osteoblast differentiation state and species differences [Owen et al., 1991]. In addition to the well-documented nuclear actions of 1α,25-$(OH)_2D_3$ in regulating gene expression, it is now recognized that this secosteroid hormone rapidly activates nongenomic signal transduction pathways [Nemere et al., 1984; Baran and Milne, 1986; Lieberherr, 1987; Baran et al., 1991; Baran and Sorensen, 1994].

Recently, several vitamin D analogs have been developed for both therapeutic applications [Brown et al., 1994] and for probing the cellular target sites that initiate the genomic and nongenomic actions of vitamin D [Norman et al., 1993]. 1α,25-dihydroxyvitamin D_3-3β-bromoacetate (1,25-$(OH)_2$-BE) is an analog of 1α,25-$(OH)_2D_3$

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in which the 3β-hydroxyl group of 1α,25-$(OH)_2D_3$ has been modified with a chemically reactive α-bromoester [Ray et al., 1994]. The affinity-labeling property of this synthetic ligand [Ray et al., 1996] should aid in the accurate definition of the hormone-binding domain(s) of various macromolecules. Because of its potential usefulness as an affinity-labeling reagent for vitamin D target sites in cells, the present study was undertaken to compare the genomic and nongenomic actions of 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE on parameters of osteoblast proliferation and differentiation.

METHODS

Chemicals and Supplies

The affinity-labeling analog 1,25-$(OH)_2$-BE (see Fig. 1) was synthesized as recently described [Ray et al., 1994]. 1α,25-$(OH)_2D_3$ was the kind gift of Dr. Milan Uskokovic (Hoffmann La Roche, Nutley, NJ). [Methyl ^{3}H]-thymidine (20 μCi/mmol) was purchased from New England Nuclear (Boston, MA). [^{32}P]-deoxycytidine triphosphate (dCTP) was purchased from Amersham (Arlington Heights, IL). The cell line ROS 17/2.8 was the generous gift of Dr. Gideon Rodan (Merck, Sharp, and Dohne, West Point, PA). The cell line ROS 24/1 was the kind gift of Dr. Mark Haussler (University of Arizona, Tucson, AR). Quin-2 AM was obtained from Calbiochem and fetal bovine serum (FBS, lot #45DO823, heat inactivated) from Gibco Laboratories (Grand Island, NY). Plastic tissue culture supplies were from Corning (Cambridge, MA). All other chemicals and cell culture reagents were from Sigma Chemical Company (St. Louis, MO) unless specified.

Osteosarcoma Cell Cultures

ROS 17/2.8 cells were grown to confluency in culture medium consisting of Dulbecco's Modified Minimum Essential medium: Ham's F12 medium (DME:F12, 1:1) plus 7% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). ROS 24/1 cells were grown in DME:F12 containing 10% FBS and antibiotics. Both cell lines were confirmed by DNA fluorochrome staining method (CELL shipper kit, Bionique Laboratories, Saranac Lake, NY) to be free of mycoplasma contamination. Cells were utilized between the fifth and 15th passage for all studies. At the start of each experiment, ROS 17/2.8 cells were harvested by trypsinization and seeded at a density of 2×10^4 cells/ml onto six-well plates in DME:F12 containing 7% FBS and antibiotics (growth medium). Cells were allowed to attach for 24 hr, then the medium was replaced with fresh growth medium containing 1α,25-$(OH)_2D_3$, 1,25-$(OH)_2$-BE, or ethanol vehicle (final ethanol concentration of 0.15%). The growth medium containing freshly added treatment agents was replaced again at 72, 96, 120, and 144 hr of culture. Cell morphology was assessed by phase contrast microscopy.

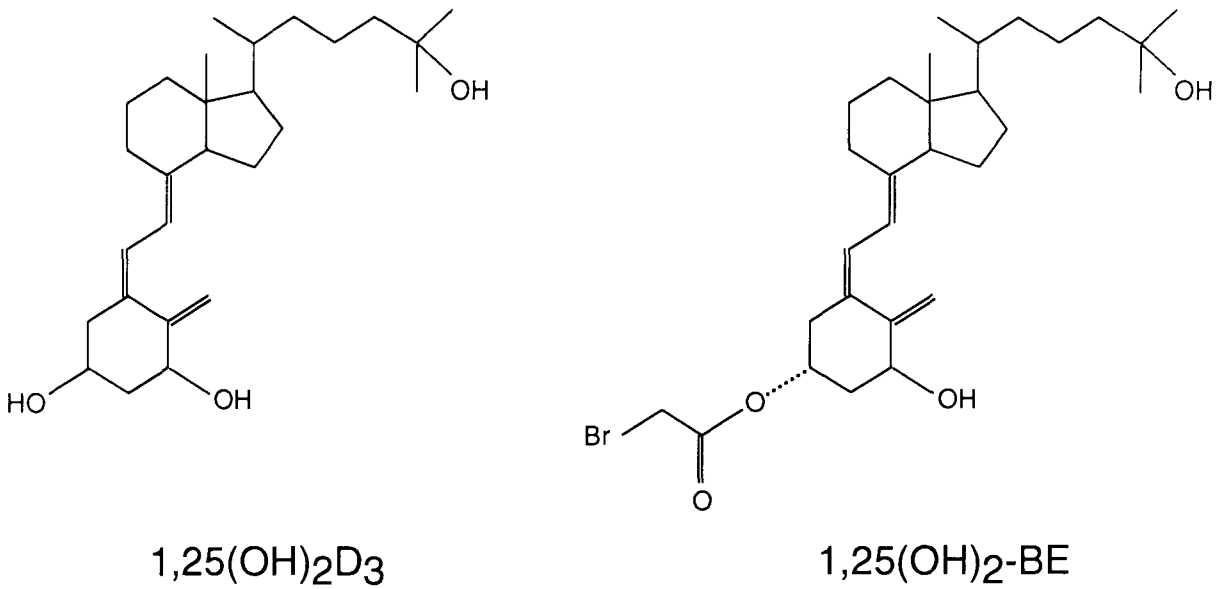

Fig. 1. Chemical structure of 1α,25-dihydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3-3β-bromoacetate.

Cell Proliferation Assays

For determination of the rate of DNA synthesis in control and treated ROS 17/2.8 cells, cultures were analyzed after 72, 96, 120, and 144 hr of treatment. During the final 4 hr of each time point, cells were incubated with 1 μCi/ml [^{3}H]-thymidine. Incorporation of the label into DNA was assessed by measuring the radioactivity in the acid-insoluble fraction of the cell layers. Briefly, the labeled cells were washed extensively with cold phosphate-buffered saline, pH 7.4, followed by fixation in methanol. Cells were then washed three times with ice-cold 10% trichloroacetic acid followed by a 30 min incubation at 60°C with 0.3 N NaOH/1% sodium dodecylsulfate (SDS). The radioactivity in the acid-insoluble extract was determined by scintillation counting. Replicate cultures were measured for each determination.

Cell proliferation was also assessed at multiple time points by determining total cell number per culture well for control and treated ROS 17/2.8 cells. After trypsinization, cells were counted using a hemacytometer and microscope, and values expressed as total number of cells/well.

Osteocalcin Secretion

Conditioned media from control and treated ROS 17/2.8 cells were harvested after 72, 96, 120, and 144 hr. Osteocalcin (OC) was measured in triplicate samples using a radioimmunoassay kit specific for rat OC (Biomedical Technologies, Stoughton, MA) and expressed as ng OC/ml conditioned culture medium.

Alkaline Phosphatase Activity

Alkaline phosphatase (AP) activity was measured in ROS 17/2.8 cells treated for 72 and 96 hr in culture. Replicate wells were analyzed for each determination. The cell layers were washed in ice-cold Tris-buffered saline, pH 7.4, and solubilized in distilled water containing 0.1% SDS. Cells were further lysed by repeated freezing and thawing. AP activity was measured with a Sigma assay kit (#104-LL) utilizing p-nitrophenyl phosphate as the enzyme substrate. Protein concentrations were measured in the same cell lysates using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Specific enzyme activity was expressed as μmol product/min/mg total culture protein.

RNA Isolation and Analysis

Total cellular RNA was isolated from ROS 17/2.8 cells after 48 hr of treatment with 1α,25-$(OH)_2D_3$ or 1,25-$(OH)_2$-BE (1×10^{-8} M). Cell layers were lysed and total RNA was isolated by the guanidinium thiocyanate extraction method [Chomczynski and Sacchi, 1987], using the reagent RNA_{ZOL} B (Biotecx, Houston, TX). The RNA (10 μg/lane) was fractionated by electrophoresis in a 1.2% agarose/1.8% formaldehyde gel and then transferred to nitrocellulose membrane (Duralose-UV, Stratagene, La Jolla, CA). Ethidium bromide staining was used to demonstrate that equivalent amounts of RNA were loaded per lane. The UV-cross-linked membrane was hybridized with two cDNA probes simultaneously: rat AP [Noda et al., 1987] and rat OC [Lian et al., 1989], kindly provided by Dr. Jane Lian, University of Massachusetts Medical Center. Probes were labeled with [^{32}P]-dCTP by the random primer method [Feinberg and Vogelstein, 1983]. Membrane prehybridization (6 hr) and hybridization (18 hr) were performed at 42°C. The membrane was then washed with increasing stringency, with the final wash in 0.1× SSPE/0.5% SDS for 30 min at 55°C. (1× SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA.) The hybridization signals were visualized by autoradiography at two exposure times.

Determination of Intracellular Calcium Levels by Quin-2 Fluorescence

Intracellular [Ca^{2+}] was measured in ROS 24/1 cells as described previously [Baran et al., 1991]. Quin-2 AM (1 μmol in 10 μl dimethylsulfoxide) was added to 1×10^8 cells suspended in 10 ml of HEPES-buffered salt solution (HBSS) containing 0.15 gm bovine serum albumin. Cells were incubated at 37°C for 30 min and pelleted at 50 × *g*. Quin-2 AM–loaded cells were then resuspended in HBSS and incubated with 1α,25-$(OH)_2D_3$ (2×10^{-8} M), 1,25-$(OH)_2$-BE (2×10^{-8} M), or ethanol vehicle. Fluorescence was measured after 5 min of treatment. Cellular fluorescence was determined using a 650-10s scanning fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT).

Statistics

Data were expressed as the mean ± SEM. Statistical analysis was performed by analysis of

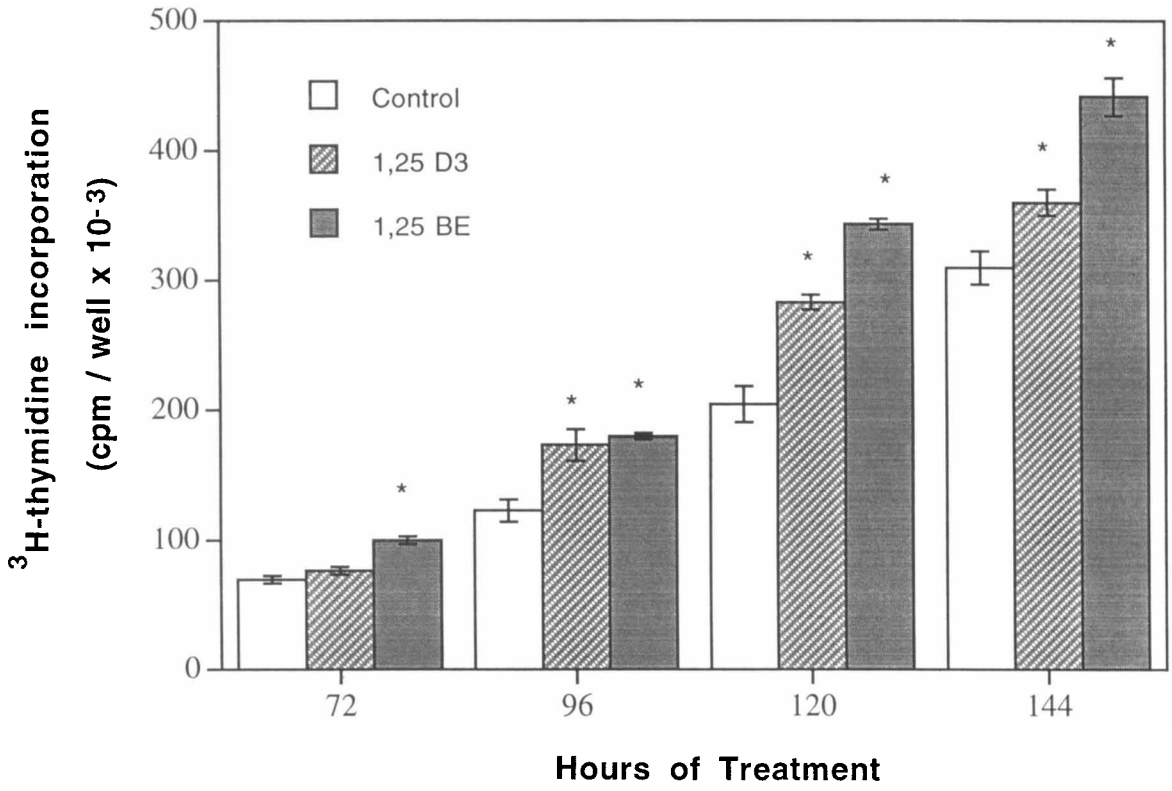

Fig. 2. Effects of 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE (1×10^{-8} M) on DNA synthesis assessed by [^{3}H]-thymidine incorporation into DNA in ROS 17/2.8 cells. Data are expressed as counts per minute incorporated per 35 mm well during a 4 hr incubation at the designated time points. Data shown are from one of four representative experiments, each with similar results. Data are expressed as mean ± SEM for four determinations at each time point. *Indicates a significant increase compared to untreated cells ($\alpha < 0.05$ or better level of significance, ANOVA).

variance and statistical differences were determined using Tukey's Studentized Range Test.

RESULTS

At 1×10^{-8} M, 1α,25-$(OH)_2D_3$ increased the incorporation of [^{3}H]-thymidine into DNA of ROS 17/2.8 cells. This effect was apparent at 72 hr of treatment and continued at each of the following 24 hr intervals assessed (Fig. 2). Treatment with 1,25-$(OH)_2$-BE (1×10^{-8} M) caused a significant increase in [^{3}H]-thymidine incorporation above that of 1α,25-$(OH)_2D_3$ at each time point measured (Fig. 2). This increase in DNA synthesis by both treatment agents was accompanied by increased proliferation of ROS 17/2.8 cells. As shown in Table I, 1α,25-$(OH)_2D_3$ (1×10^{-8} M) increased cell number 135% above control after 96 hr of exposure, and 52% above control after 144 hr of treatment. The bromoester analog stimulated cell proliferation to an even greater extent at each time point examined (171% increase at 96 hr and 102% increase at 144 hr).

TABLE I. Effect of 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE on ROS 17/2.8 Cell Growth†

Hours in culture	Total cells/well × 10^{-4}		
	Control	1α,25-$(OH)_2D_3$	1,25-$(OH)_2$-BE
24	1.2 ± 0.2	—	—
96	14.0 ± 0.8	33.0 ± 0.7*	38.0 ± 2.8*
144	99.0 ± 0.2	150.0 ± 16.0*	200.0 ± 15.0*

†ROS 17/2.8 cultures were treated as described in Figure 2. At the times indicated, cell counts were performed in parallel cultures. Data are the mean ± SEM of six determinations for each time point.
*Indicates significant increase compared to control cultures, $P < 0.05$ or less.

Treatment of ROS 17/2.8 cells with the vitamin D compounds altered the appearance of the cells from small and compact cuboidal to a more flattened and elongated shape. These morphological changes were apparent in both 1α,25-

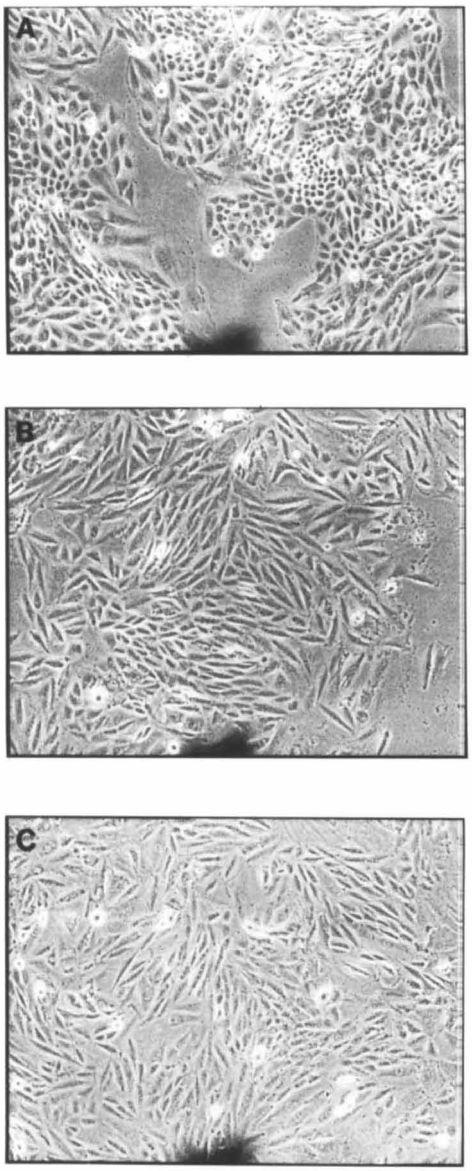

Fig. 3. Morphology of (A) control, (B) 1α,25-$(OH)_2D_3$-, and (C) 1,25-$(OH)_2$-BE-treated ROS 17/2.8 cells. Cells were plated at low density and treated 24 hr later with ethanol vehicle (control), or with one of the two vitamin D compounds (1×10^{-8} M). Phase contrast photomicrographs (original magnification: 100×) were taken after 120 hr of treatment.

$(OH)_2D_3$- and 1,25-$(OH)_2$-BE-treated cultures by 96 hr, and were still evident after 144 hr of treatment (Fig. 3).

The induction of OC and AP mRNA in response to the treatment agents was compared and is shown in Figure 4. After ROS 17/2.8 cells had been exposed for 48 hr to 1α,25-$(OH)_2D_3$ or 1,25-$(OH)_2$-BE at 1×10^{-8} M, OC mRNA expression was increased above basal expression, while AP mRNA levels were slightly decreased.

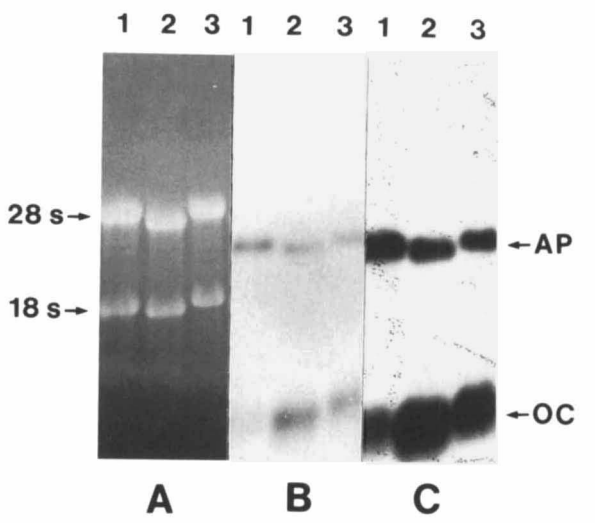

Fig. 4. Northern blot analysis of total cellular RNA (10 μg lane) prepared from control (**lane 1**), 1α,25-$(OH)_2D_3$- (**lane 2**), and 1,25-$(OH)_2$-BE-treated (**lane 3**) ROS 17/2.8 cells. Cultures were treated with 1×10^{-8} M agent and harvested 48 hr later. After ethidium bromide staining (**A**), the gel was transferred to nitrocellulose membrane and hybridized simultaneously with cDNA probes for rat AP and rat OC; **B** represents the hybridized membrane subjected to autoradiography at room temperature for 5 hr; **C** is the same membrane autoradiographed for 8 hr at −70°C, using an intensifying screen.

Although 1α,25-$(OH)_2D_3$ increased OC mRNA, it did not significantly increase OC secretion. Exposure to 1α,25-$(OH)_2D_3$ ($1 \times \mathbf{10^{-8}}$ M) for 72, 120, and 144 hr resulted in slight, but not significant, increases in osteocalcin protein secreted into the medium (Fig. 5). In contrast, at the same concentration, 1,25-$(OH)_2$-BE significantly stimulated OC secretion above control levels at all four time points examined.

The clonal ROS 17/2.8 cell line utilized in our laboratory had an AP-specific activity of 2.04 μmol/min/mg total cellular protein. Figure 6 shows the effect of 1α,25-$(OH)_2D_3$ vs 1,25-$(OH)_2$-BE at 1×10^{-8} M on AP activity. Cultures were examined after 72 and 96 hr of treatment. At both time points, 1α,25-$(OH)_2D_3$ decreased AP-specific activity marginally. The bromoester analog reduced AP-specific activity to a greater extent than the natural ligand at both time points examined, with a 25% reduction below control levels after 96 hr of treatment ($\alpha < 0.05$, by ANOVA).

In order to evaluate the effects of 1,25-$(OH)_2$-BE on osteoblast responses which do not involve gene activation, the clonal rat osteosarcoma cell line ROS 24/1 was utilized. Exposure to either 1α,25-$(OH)_2D_3$ or 1,25-$(OH)_2$-BE at a concentration of 2×10^{-8} M rapidly increased

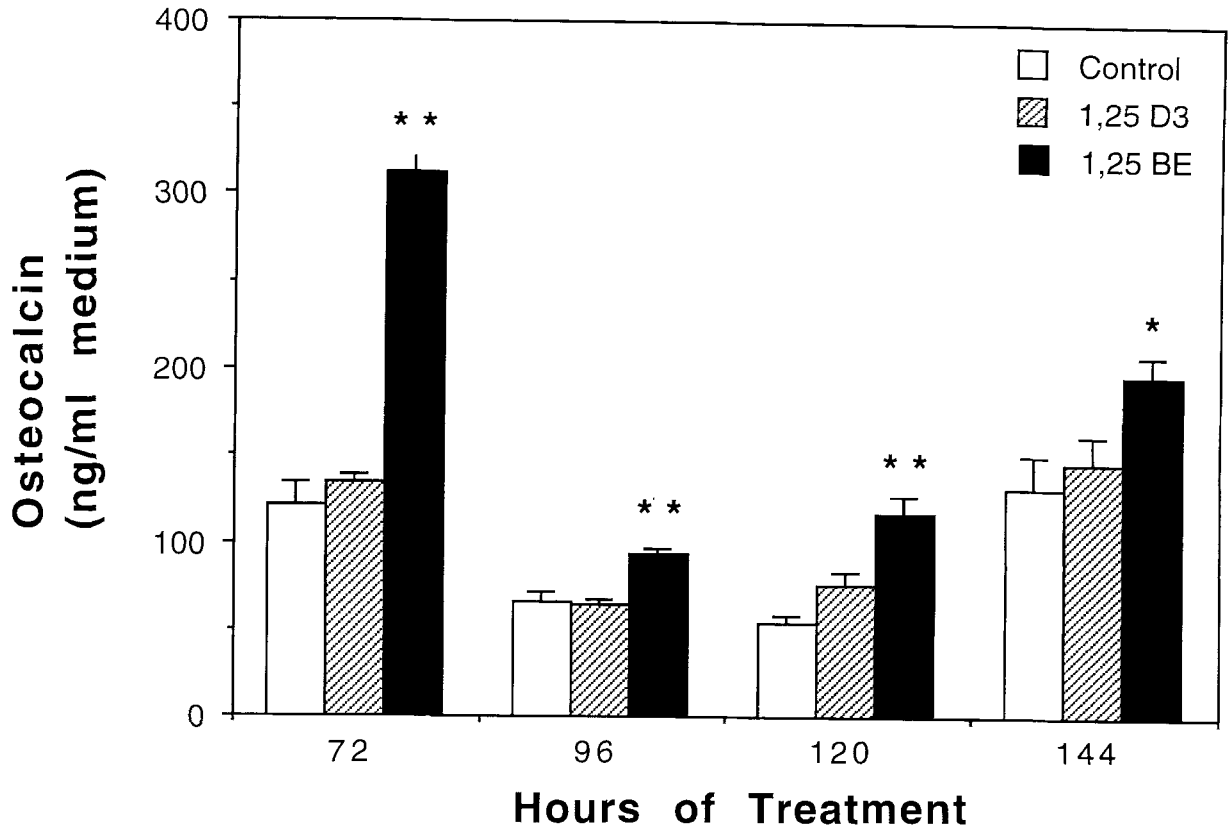

Fig. 5. Effects of 1α,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-BE treatment on osteocalcin production by ROS 17/2.8 cells. Cultures were treated as described in Figure 2. The conditioned media were collected and assayed for osteocalcin content by radioimmunoassay. Data represent the mean ± SEM of triplicate determinations from one of three representative experiments. *Indicates a significant increase compared to control. **Indicates a significant increase compared to control and 1α,25-$(OH)_2D_3$ values ($\alpha < 0.05$ or better level of significance).

intracellular calcium in this osteoblastic cell line, as shown in Table II.

DISCUSSION

We have shown that the vitamin D_3 affinity-labeling analog 1,25-$(OH)_2$-BE enhances the proliferation of ROS 17/2.8 cells. The mitogenic effect of this analog is accompanied by distinct changes in cell morphology and a decrease in AP-specific enzyme activity. Both effects are comparable to those of 1α,25-$(OH)_2D_3$. The steady-state mRNA level of AP displays a moderate decrease after 48 hr of exposure to 1,25-$(OH)_2$-BE, while the mRNA level of OC is increased during treatment. OC secretion into the culture medium increases significantly as a result of analog treatment. We have further demonstrated that 1,25-$(OH)_2$-BE rapidly increases cytosolic calcium in ROS 24/1 cells. The biological responses of this novel vitamin D analog on the two rat osteosarcoma cell lines are similar to treatment with 1α,25-$(OH)_2D_3$, yet are of greater magnitude than those of the natural hormone. We speculate that this is due to the ability of the synthetic analog to bind covalently to cellular ligand binding sites. This is supported by a recent report demonstrating the specificity of labeling of the classic nuclear vitamin D receptor with 1,25-$(OH)_2D_3$-[^{14}C]BE [Ray et al., 1996]. The differences we observe in cellular response between the natural ligand and the bromoester analog could be due to the stronger affinity of 1,25-$(OH)_2$-BE to the nuclear vitamin D receptor. This greater binding affinity may have resulted in the prolonged effect of the analog on OC secretion (Fig. 5) and AP-specific activity (Fig. 6) in ROS 17/2.8 cells. Analysis of additional parameters such as histone, collagen type I, and osteopontin expression may reveal that 1,25-$(OH)_2$-BE has more potent effects on these osteoblast functions than the natural ligand.

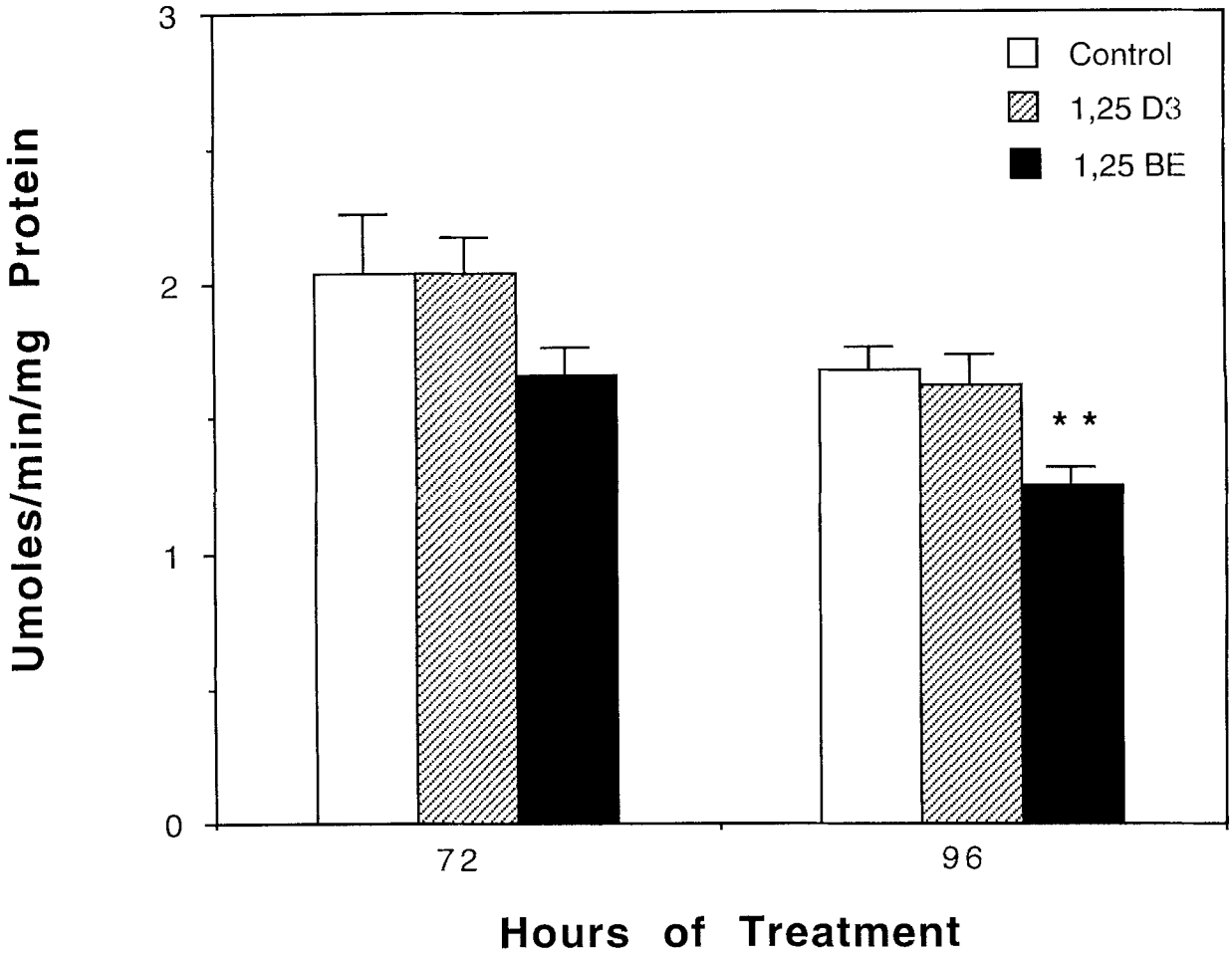

Fig. 6. Alkaline phosphatase biochemical activity in control, 1α,25-$(OH)_2D_3$-, or 1,25-$(OH)_2$-BE-treated ROS 17/2.8 cells. Cells were treated as described in Figure 3. AP activity is expressed as μmoles product/minute/mg total cell protein. Values represent the mean ± SEM of four determinations. **Indicates a significant difference from control (ANOVA, $\alpha < 0.05$).

TABLE II. Effect of 1α,25-$(OH)_2D_3$ or 1,25-$(OH)_2$-BE on Intracellular Calcium in ROS 24/1 Cells*

	[Ca++] (nM)	Increase (%)
Control	90.0 ± 15.0	—
1α,25-$(OH)_2D_3$ (2×10^{-8} M)	126.0 ± 25.0	140
1,25-$(OH)_2$-BE (2×10^{-8} M)	129.0 ± 19.0	143

*Cytosolic calcium was measured by Quin 2 fluorescence after a 5 min exposure of ROS 24/1 cells to 1α,25-$(OH)_2D_3$ or 1,25-$(OH)_2$-BE. Values represent the mean ± SEM of six determinations for each data point.

Numerous studies have demonstrated that 1α,25-$(OH)_2D_3$ treatment alters the growth and differentiation of normal and transformed osteoblastic cells [Majeska and Rodan, 1982; Dokoh et al., 1984; Beresford et al., 1986; Marie et al., 1990]. Both proliferative and antiproliferative effects have been reported.

Our findings with the ROS 17/2.8 cell line are consistent with those of Majeska and Rodan [Majeska and Rodan, 1982]. They reported a dose-dependent reduction in AP-specific activity when 1α,25-$(OH)_2D_3$ (10^{-12} M–10^{-9} M) was added to "mature" ROS 17/2.8 cultures maintained in the presence of 2% serum. Additionally, hormone treatment caused a small but significant increase in cell number. These osteosarcoma cells were defined as phenotypically mature by the exhibition of high AP-specific activity. The ROS 17/2.8 cells used in the present study were cultured under conditions that yield a baseline AP activity of 2.04 μmol/min/mg cell protein (high specific activity) when

seeded at 2×10^4 cells/ml and assayed after 4 d in culture. Intriguingly, the decrease in AP activity and increase in cell number reported previously [Majeska and Rodan, 1982] were obtained in high cell density cultures, whereas our findings on cell proliferation and AP activity were obtained from subconfluent cultures. It is evident in Figure 3 that control, 1α,25-$(OH)_2D_3$-treated, and 1,25-$(OH)_2$-BE-treated cells were subconfluent even after 120 hr of treatment. It has been hypothesized that the ROS 17/2.8 cell line contains subpopulations of cells possessing different degrees of phenotypic differentiation [Manolagas et al., 1983]. These investigators have observed a dose-dependent increase in AP activity in ROS 17/2.8 cells treated with 1α,25-$(OH)_2D_3$ (10^{-12} M–10^{-7} M) in the presence of serum. The discrepancies in the reported effects of 1α,25-$(OH)_2D_3$ on the differentiation state of ROS 17/2.8 cells could be explained by the existence of a number of cell line subclones. Additionally, the cell line is maintained with varying concentrations of fetal bovine serum. Serum contains a complex mixture of growth factors and other defined and undefined components whose presence and concentration vary with each commercial preparation, and most likely affect the growth rate and differentiation state of the ROS cell lines. Nevertheless, the present study shows similar effects of the two vitamin D compounds on growth, morphology, mRNA expression, and protein secretion.

Nongenomic rapid actions of 1α,25-$(OH)_2D_3$ have been reported in a wide variety of tissue and cell types [de Boland and Nemere, 1991]. We have shown that ROS 24/1 cells rapidly respond to the 1,25-$(OH)_2$-BE analog with an increase in cytosolic calcium, in a manner identical to that of 1α,25-$(OH)_2D_3$. It has been postulated that 1α,25-$(OH)_2D_3$ exerts its rapid actions through a membrane-associated receptor that differs from the classic nuclear vitamin D receptor [Baran et al., 1994; Nemere and Szego, 1981]. The ability of 1,25-$(OH)_2$-BE to affinity label cellular target sites and to mediate the actions of vitamin D suggests that this novel ligand will be useful in labeling and identifying the ligand binding sites involved in the signaling pathways mediating the genomic and nongenomic effects of vitamin D.

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