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Hybrid Structural Analogues of 1,25-$(OH)_2D_3$ Regulate Chondrocyte Proliferation and Proteoglycan Production as Well as Protein Kinase C Through a Nongenomic Pathway

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Abstract 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ mediate their effects on chondrocytes through the classic vitamin D receptor (VDR) as well as through rapid membrane-mediated mechanisms which result in both nongenomic and genomic effects. In intact cells, it is difficult to distinguish between genomic responses via the VDR and genomic and nongenomic responses via membrane-mediated pathways. In this study, we used two hybrid analogues of 1,25-$(OH)_2D_3$ which have been modified on the A-ring and C,D-ring side chain (1α-(hydroxymethyl)-3β-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3 (analogue MCW-YA = 3a) and 1β-(hydroxymethyl)-3α-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3 (analogue MCW-YB = 3b) to examine the role of the VDR in response of rat costochondral resting zone (RC) and growth zone (GC) chondrocytes to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. These hybrid analogues are only 0.1% as effective in binding to the VDR from calf thymus as 1,25-$(OH)_2D_3$. Chondrocyte proliferation ([^{3}H]-thymidine incorporation), proteoglycan production ([^{35}S]-sulfate incorporation), and activity of protein kinase C (PKC) were measured after treatment with 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, or the analogues. Both analogues inhibited proliferation of both cell types, as did 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. Analogue 3a had no effect on proteoglycan production by GCs but increased that by RCs. Analogue 3b increased proteoglycan production in both GC and RC cultures. Both analogues stimulated PKC in GC cells; however, neither 3a nor 3b had an effect on PKC activity in RC cells. 1,25-$(OH)_2D_3$ and 3a decreased PKC in matrix vesicles from GC cultures, whereas plasma membrane PKC activity was increased, with 1,25-$(OH)_2D_3$ having a greater effect. 24,25-$(OH)_2D_3$ caused a significant decrease in PKC activity in matrix vesicles from RC cultures; 24,25-$(OH)_2D_3$, 3a, and 3b increased PKC activity in the plasma membrane fraction, however. Thus, with little or no binding to calf thymus VDR, 3a and 3b can affect cell proliferation, proteoglycan production, and PKC activity. The direct membrane effect is analogue-specific and cell maturation–dependent. By studying analogues with greatly reduced affinity for the VDR, we have provided further evidence for the existence of a membrane receptor(s) involved in mediating nongenomic effects of vitamin D metabolites. J. Cell. Biochem. 66:457–470, 1997. © 1997 Wiley-Liss, Inc.

Key words: vitamin D; analogue; chondrocytes; nongenomic; differentiation; 1,25-$(OH)_2D_3$; 24,25-$(OH)_2D_3$; proteoglycan; protein kinase C

Vitamin D metabolites have been shown to use two major pathways to mediate their effects on cells. According to the classic pathway, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ traverse the cell membrane and bind to the vitamin D receptor

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(VDR) in the cytosol. Once the ligand-receptor complexes are translocated to the nucleus, they interact with various transcription factors to regulate genomic events, ultimately affecting gene expression [Pike, 1992; Boyan et al., 1992]. $1,25\text{-(OH)}_2D_3$ has also been shown to regulate mRNA stability [Kyeyune-Nyombi et al., 1991]. More recently, a second pathway has emerged, involving rapid membrane events generally associated with peptide hormone stimulation of transmembrane receptors [Norman and Collins, 1996]. $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ have been shown to act directly on the plasma membrane of target cells, causing rapid changes in calcium flux [Yukihiro et al., 1994; Zhou et al., 1992; Langston et al., 1990; Farach-Carson et al., 1991], fatty acid metabolism [Schwartz et al., 1990, 1992; Swain et al., 1992; Matsumoto et al., 1981; Rasmussen et al., 1982], phospholipid metabolism [Bourdeau et al., 1990; Tang et al., 1987; Wali et al., 1990; Lieberherr et al., 1989], membrane fluidity [Swain et al., 1993], and protein kinase C (PKC) activity [Sylvia et al., 1993, 1996]. These rapid actions may modulate nongenomic events, or they may culminate in downstream genomic events.

When studying rapid responses to vitamin D metabolites in intact cells, it is difficult to definitively rule out a contribution of the VDR to the mechanism. Rapid translocation of the VDR to the plasma membrane of rat kidney cells after exposure to $1,25\text{-(OH)}_2D_3$ has been observed [Kim et al., 1994, 1996], suggesting that it acts as an acceptor for the vitamin D metabolite as it traverses the inner leaflet of the membrane. Subsequent studies showing rapid $1,25\text{-(OH)}_2D_3$–dependent intracellular calcium fluxes supported the hypothesis that the VDR could mediate rapid nongenomic actions [Kim et al., 1994, 1996]. The phenomenon of transcaltachia, the rapid movement of Ca ions across the intestinal plasma membrane, is also sensitive to $1,25\text{-(OH)}_2D_3$, but studies using analogues of this vitamin D metabolite with low binding affinity for the VDR indicate that the classic cytosolic receptor is not involved. Rather, transcaltachia appears to result from the specific interaction of $1,25\text{-(OH)}_2D_3$ with a membrane receptor [Nemere et al., 1994], resulting in rapid regulation of voltage-gated Ca ion channels in osteoblasts as well as intestinal epithelial cells.

Studies in our laboratory using plasma membranes and matrix vesicles isolated from growth plate chondrocyte cultures indicate that $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ interact in a specific manner with the membrane itself to initiate several signal transduction pathways [Sylvia et al., 1993, 1996; Swain et al., 1993]. Moreover, these events are specific to the state of endochondral maturation of the cartilage from which the cells were originally derived and to the type of membrane being examined. This implies that receptors for specific vitamin D metabolites exist in the membranes.

These experiments using isolated membranes do not represent a fortuitous observation, without relevance to the intact cell where rapid recycling of the receptor is possible. Matrix vesicles are located in the territorial matrix of chondrocytes and osteoblasts [Boyan et al., 1994a,b; Schwartz et al., 1988a] and can be isolated from cultures in intact form, right-side out [Schwartz et al., 1988b]. Whereas the vitamin D metabolite–responsive PKC isoform in plasma membranes is PKCα, in matrix vesicles it is PKCζ [Sylvia et al., 1996]. This differential distribution of PKC isoforms provides at least one mechanism by which vitamin D metabolites can elicit one response in plasma membranes and another opposite effect in matrix vesicles. Matrix vesicles contain neither RNA nor DNA, so at least for this extracellular organelle a genomic interpretation of the data is not valid.

Recently, we reported studies using two analogues of $1,25\text{-(OH)}_2D_3$ which had been modified on the A ring, 1α-(hydroxymethyl)-3β,25-dihydroxyvitamin D_3 (2a) and 1β-(hydroxymethyl)-3α,25-dihydroxyvitamin D_3 (2b) [Greising et al., 1996, in press; Posner and Dai, 1993; Posner et al., 1992]. Despite their reduced binding capacity to the VDR to less than 0.1% that of $1,25\text{-(OH)}_2D_3$, these analogues caused a reduction in chondrocyte proliferation [Greising et al., 1996, in press], indicating that this response to vitamin D is not VDR-dependent. In addition, by use of these analogues in our chondrocyte model, we have also shown that at least some of the effect of the analogues is via nongenomic mechanisms; this membrane-associated nongenomic response is cell maturation–dependent; and the membrane effect may involve specific receptors.

In order to obtain a more precise picture of how conformational changes of $1,25\text{-(OH)}_2D_3$ modulate chondrocyte proliferation, differentiation, matrix production, and transmembrane

signaling mechanisms, we used two new hybrid analogues of the vitamin D metabolite which contain modifications on both the A-ring and C,D-ring side chain, analogue MCW-YA (3a) and its stereoisomer MCW-YB (3b) [Posner et al., 1994, 1995] (Fig. 1). Both hybrid analogues exhibit very high antiproliferative activity. Although they both display less than 0.1% of the binding affinity of 1,25-$(OH)_2D_3$ to the calf thymus VDR, one report has shown that the analogues still retain transcriptional activity through the VDR [Peleg et al., 1996]. The hybrid analogues stimulate instantaneous calcium channel opening, a nongenomic process in rat osteosarcoma cells [Posner et al., 1994]. The results of the present study demonstrate the existence of a separate membrane receptor and show that these vitamin D hybrid analogues have potential as pharmaceuticals for treatment of specific vitamin D–related diseases which do not involve the VDR. Moreover, they show that the state of cell maturation modulates the membrane response.

MATERIALS AND METHODS

Synthesis of Vitamin D Analogues

Two hybrid analogues of 1,25-$(OH)_2D_3$ were used for the described studies. Analogue MCW-YA (3a) (1α-(hydroxymethyl)-3β-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3) and analogue MCW-YB (3b) (1β-(hydroxymethyl)-3α-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3) (Fig. 1) were synthesized by convergent coupling of 1-hydroxymethyl A-ring phosphine oxide with modified C,D-ring ketones [Posner et al., 1992, 1993a, 1994, 1995]. Small samples of these hybrid analogues are available from GHP for further biological evaluation.

Each vitamin D_3 hybrid analogue was tested at 10^{-6} to 10^{-9} M, which includes a range of both physiological and pharmacological doses of the active metabolites of vitamin D_3 (1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$, gifts from Dr. Milan Uskokovic, Hoffman-LaRoche, Nutley, NJ). The metabolites and analogues were dissolved in ethanol. These stock solutions were diluted at least 1:5,000 v/v with Dulbecco's modified Eagle's medium (DMEM) (Grand Island Biological, Grand Island, NY) prior to adding to the culture medium to minimize any toxic effects. Ethanol at the same concentration was used as an internal control.

Chondrocyte Cultures

The culture system has been previously described in detail [Boyan et al., 1988a,b]. Resting zone (reserve zone) and growth zone (prehypertrophic and upper hypertrophic zones) chondrocytes were isolated from the costochondral cartilage of 125 g male Sprague-Dawley rats. The cells were seeded into T-75 flasks at densities of 10,000 cells/cm^2 for resting zone chondrocytes and 25,000 cells/cm^2 for growth zone chondrocytes and were cultured in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml vitamin C in an atmosphere of 5% CO_2 and 100% humidity at 37°C. The culture media were replaced after 24 h and then at 72 h intervals. At confluence, the cells were subcultured at the same seeding densities. Third passage, confluent cultures were subpassaged into 24-well microtiter plates, grown to confluence, and then used for the experiments. Prior studies have demonstrated a retention of differential phenotype with respect to vitamin D response through this number of passages in culture [Schmitz et al., 1996; Dean et al., 1996; Boyan et al., 1988a,b, 1989, 1994a,b; Schwartz et al., 1988a,b; Sylvia et al., 1993, 1996; Swain et al., 1993].

Isolation of Plasma Membrane and Matrix Vesicle Fractions

Chondrocyte plasma membranes and extracellular matrix vesicles were isolated from confluent cultures by digestion with 1% trypsin, as described previously [Boyan et al., 1988b]. Cells were separated by centrifugation from the trypsin-digested matrix and resuspended in Hank's balanced salt solution. Chondrocytes were then homogenized and plasma membranes prepared according to the method of Fitzpatrick et al. [1969]. The trypsin digest supernatant was centrifuged again at 21,000g for 10 minutes to pellet cell debris, including mitochondria and endoplasmic reticulum. The resulting supernatant was centrifuged at 100,000g for 1 h to pellet matrix vesicles. Protein content [Smith et al., 1985] and alkaline phosphatase activity [Bretaudiere and Spillman, 1984], using p-nitrophenylphosphate as substrate at pH 10.2, were determined for each membrane preparation.

Matrix vesicles isolated in this manner typically exhibit greater than twofold enrichment of alkaline phosphatase specific activity when

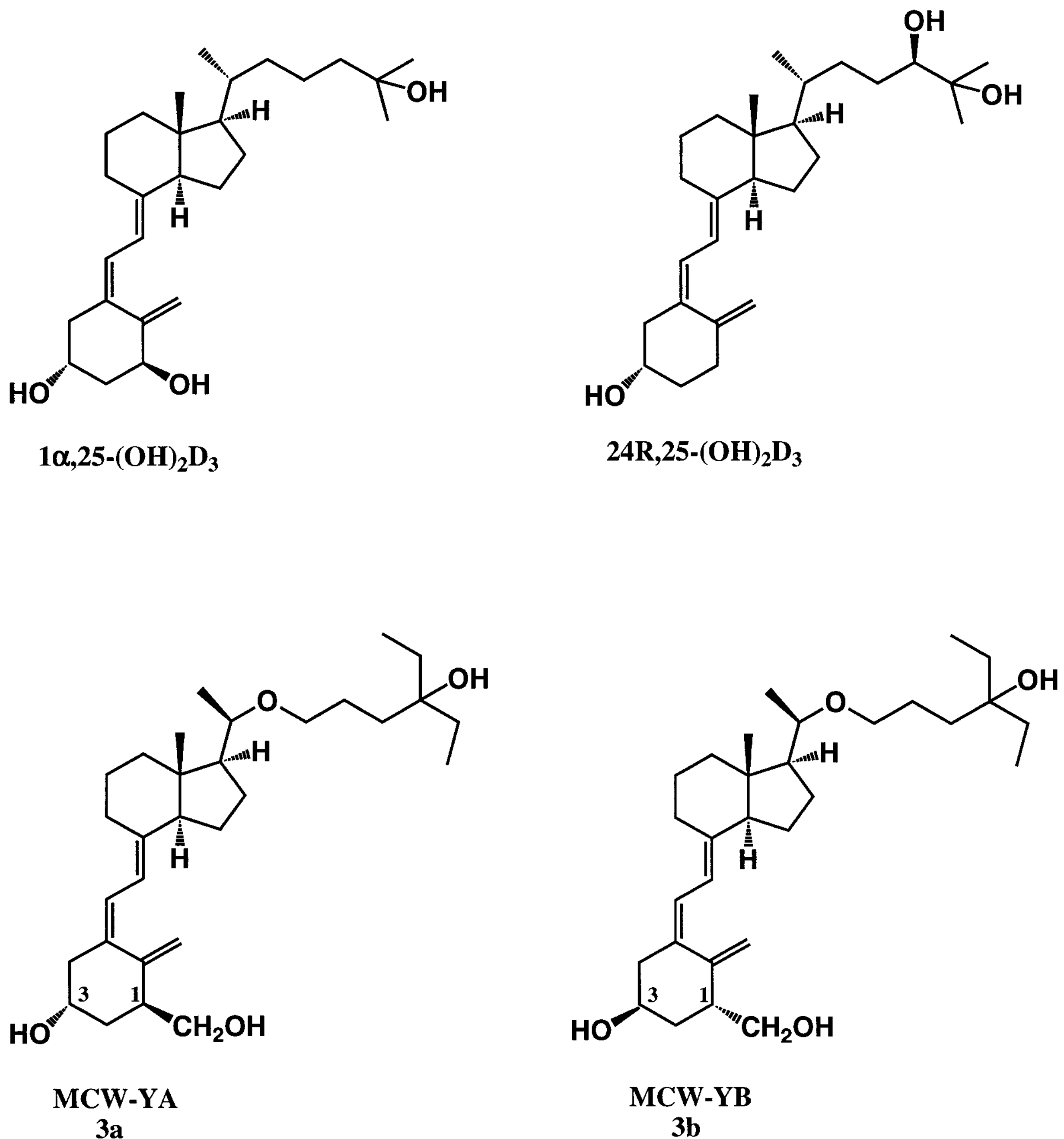


Fig. 1. Chemical structures of the different vitamin D analogues used in this study. The top shows the chemical structures for 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$. The chemical name for analogue MCW-YA (3a), shown in the lower left, is 1α-(hydroxymethyl)-3β-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3. The chemical name for analogue MCW-YB (3b), shown in the lower right, is 1β-(hydroxymethyl)-3α-hydroxy-20-epi-22-oxa-26,27-dihomo vitamin D_3.

compared with the plasma membranes [Schwartz et al., 1988a; Boyan et al., 1988a,b] and have a transmission electron microscopic appearance consistent with matrix vesicles in vivo [Boyan et al., 1988a]. The purity of the matrix vesicle preparations has been established previously [Schwartz et al., 1988a; Boyan et al., 1988a]. Further, since homogenization or lysing of the cultures is not necessary for matrix vesicle isolation, the matrix vesicles are intact and right-side out after isolation. They contain no DNA or RNA; therefore, any effect

directly on the matrix vesicles will not involve new gene transcription or translation.

[^{3}H]-Thymidine Incorporation

DNA synthesis was estimated by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA)–insoluble cell precipitates, as described previously [Schwartz et al., 1989]. Quiescence was induced by incubating resting zone and growth zone chondrocytes for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS and various concentrations of vitamin D metabolite or analogue. [^{3}H]-thymidine (2 μCi/ml) was added 2 h before harvest. At harvest, the cell layers were washed twice with cold phosphate buffered saline (PBS) and twice with 5% TCA and then treated with saturated TCA for 30 min. TCA-precipitable material was dissolved in 0.2 ml 1% sodium dodecyl sulfate (SDS) and the radioactivity measured by liquid scintillation spectroscopy.

[^{35}S]-Sulfate Incorporation

Proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation according to the method of O'Keefe et al. [1988]. In prior studies [Nasatzky et al., 1994], we found that the amount of radiolabeled proteoglycan released by growth zone and resting zone chondrocytes into the medium was less than 15% of the total radiolabeled proteoglycan (media and cell layer) synthesized. Therefore, we examined only the effects of hormone treatment on [^{35}S]-sulfate incorporation in the cell layer.

For assay, fourth passage growth zone and resting zone chondrocytes were grown to confluence in 24-well culture plates (Corning, Corning, NY). Twenty-four hours before harvest, fresh media containing vehicle alone or varying concentrations of vitamin D metabolite or analogue were added. Four hours prior to harvest, 50 μl DMEM containing 13 μCi/ml [^{35}S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed and the cell layers (cells and matrix) collected in two 0.25 ml portions of 0.25 M NaOH. The total volume was adjusted to 0.75 ml by the addition of 0.15 M NaCl and the sample dialyzed in a 12,000–14,000 molecular weight cut-off membrane against buffer containing 0.15 M NaCl, 20 mM Na_2SO_4, and 20 mM Na_2HPO_4, pH 7.4, at 4°C. The dialysis solution was changed until the radioactivity in the dialysate reached background levels. The protein content was then determined using a macro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The amount of [^{35}S]-sulfate incorporated was determined by liquid scintillation spectrometry and calculated as disintegrations per minute/mg protein in the cell layer.

Protein Kinase C Activity

Prior studies had shown that PKC activity in growth zone chondrocytes is increased by treatment with 1,25-$(OH)_2D_3$ but not 24,25-$(OH)_2D_3$ and that maximal response is seen by 9 min. In contrast, PKC activity in resting zone chondrocytes is affected by 24,25-$(OH)_2D_3$ but not 1,25-$(OH)_2D_3$, and maximal response is seen by 90 min [Sylvia et al., 1993]. Therefore, for the current studies, vitamin D_3 analogues and metabolites were added to growth zone chondrocytes for 9 min and to resting zone chondrocytes for 90 min. After incubation in experimental or control media, the cell layers were washed with PBS, loosened from the wells with a sterile cell scraper, and lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1% NP-40) for 30 min on ice.

Chondrocyte cell layer lysates, containing equivalent amounts of protein, were mixed for 20 min with a lipid preparation containing phorbol-12-myristate-13-acetate, phosphatidylserine, and Triton X-100 mixed micelles to provide the necessary cofactors and conditions for optimal enzyme activity [Bell et al., 1986]. To this mixture, a high-affinity myelin basic protein peptide and [^{32}P]-ATP (25 μCi/ml) were added to a final assay volume of 50 μl. Following a 10 min incubation in a 30°C water bath, samples were spotted onto phosphocellulose discs, which were then washed twice with 1% phosphoric acid and once with distilled water to remove unincorporated label prior to placement in a scintillation counter. To verify whether the kinase activity affected by the analogues was restricted to PKC activity and not due to other protein kinases, we added a specific inhibitor peptide corresponding to amino acid residues 19–36 of the PKC pseudosubstrate region to the PKC reaction tubes at a final concentration of 3 μM [Yasuda et al., 1990].

To examine the involvement of genomic mechanisms in PKC activation, we treated the chondrocyte cultures with vitamin D_3 analogues in the presence of the transcription in-

hibitor actinomycin D or the translation inhibitor cycloheximide. Growth zone chondrocytes were incubated with analogue and 0.01 mM actinomycin D or 0.1 mM cycloheximide for 9 min. Resting zone chondrocytes were incubated with analogue and 0.01 mM actinomycin D or 0.1 mM cycloheximide for 90 min. Following treatment with the inhibitors, the cells were washed with PBS and assayed for PKC activity as described above. To examine the role of nongenomic mechanisms, isolated matrix vesicles and plasma membranes were incubated directly with the vitamin D_3 metabolites or analogues and PKC activity measured as described previously [Sylvia et al., 1996].

Statistical Analysis

All data are expressed as the mean ± the standard error of the mean (SEM) of six cultures. Figures contain data from representative experiments. All experiments were performed a minimum of three times. Significant effects were determined by ANOVA and significance between groups determined using the Student's *t*-test with Bonferroni's correction for multiple comparisons. *P* values less than 0.05 were considered significant.

RESULTS

Cell Proliferation

When either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ was added to cultures of growth zone chondrocytes, a significant inhibition in [^{3}H]-thymidine incorporation was observed (Fig. 2A). The inhibition produced by 1,25-$(OH)_2D_3$ was greater than that produced by 24,25-$(OH)_2D_3$. Addition of either analogue 3a or 3b to the cells produced a dose-dependent inhibition of [^{3}H]-thymidine incorporation (Fig. 2A). The inhibition was less pronounced than that observed with 1,25-$(OH)_2D_3$ at the same concentration but comparable to that seen with 24,25-$(OH)_2D_3$. [^{3}H]-thymidine incorporation by resting zone chondrocytes was inhibited by 1,25-$(OH)_2D_3$, whereas 24,25-$(OH)_2D_3$ had no effect (Fig. 2B). Addition of 3a or 3b to the cultures resulted in a dose-dependent inhibition of [^{3}H]-thymidine incorporation that was significant over the range of 10^{-7} to 10^{-6} M.

Proteoglycan Production

When 1,25-$(OH)_2D_3$ was added to cultures of growth zone chondrocytes, a significant in-

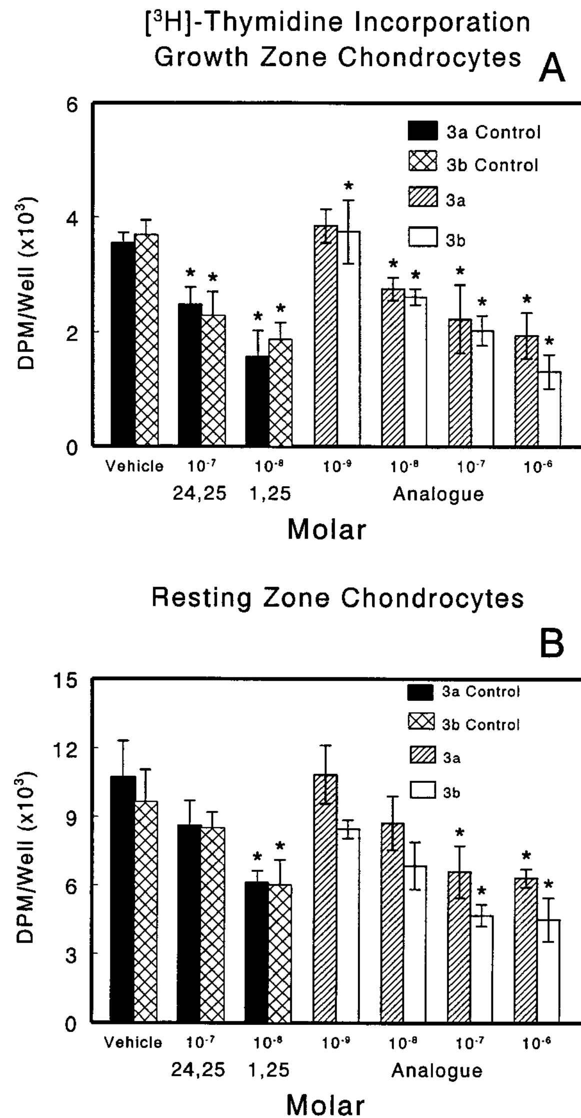


Fig. 2. The effect of vitamin D analogues 3a and 3b on [^{3}H]-thymidine incorporation by growth zone and resting zone chondrocytes. Confluent, fourth passage growth zone (**A**) and resting zone (**B**) chondrocytes were treated for 24 h with vitamin D vehicle, 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or vitamin D analogue and [^{3}H]-thymidine incorporation determined. The figure shows the results of two separate experiments, one for 3a and one for 3b, each with its own 1,25 and 24,25 and vehicle only controls. Each experiment was repeated three times. Values represent the mean ± SEM for six separate cultures. $^*P < 0.05$, significantly different from untreated control.

crease in [^{35}S]-sulfate incorporation was observed, whereas 24,25-$(OH)_2D_3$ was without effect. Similarly, analogue 3a had no effect on [^{35}S]-sulfate incorporation (Fig. 3A), while analogue 3b stimulated [^{35}S]-sulfate incorporation in a dose-dependent manner, with peak stimulation occurring in cultures treated with 10^{-7} M

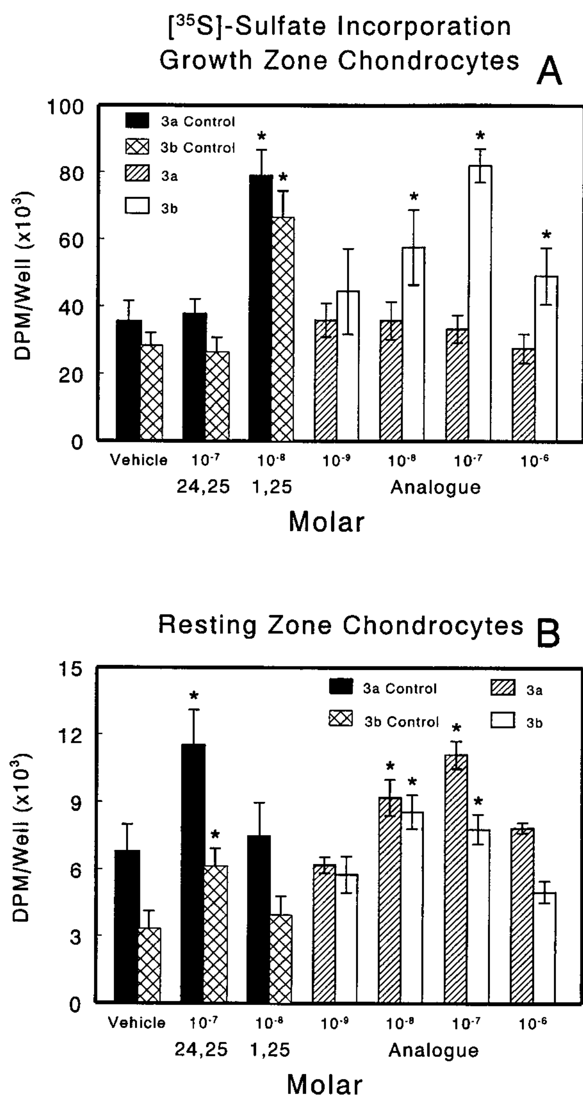


Fig. 3. The effect of vitamin D analogues 3a and 3b on [^{35}S]-sulfate incorporation by growth zone (**A**) and resting zone (**B**) chondrocytes. Confluent, fourth passage chondrocytes were treated for 24 h with vitamin D vehicle, 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or vitamin D analogue and [^{35}S]-sulfate incorporation by the cells determined. The figure shows the results of two separate experiments, one for 3a and one for 3b, each with its own 1,25 and 24,25 and vehicle only controls. Each experiment was repeated three times. Values represent the mean ± SEM for six separate cultures. *$P < 0.05$, significantly different from untreated control.

3b (Fig. 3A). When 10^{-7} M 24,25-$(OH)_2D_3$ was added to cultures of resting zone chondrocytes, a significant increase in [^{35}S]-sulfate incorporation was observed (Fig. 3B). In contrast, no effect was found with 1,25-$(OH)_2D_3$. However, both analogues stimulated [^{35}S]-sulfate incorporation in a dose-dependent manner.

Protein Kinase C Activity

Treatment of growth zone chondrocytes with 1,25-$(OH)_2D_3$ for 9 min produced a significant increase in PKC activity that was more than four times the level found in untreated cultures (Fig. 4A). In contrast, treatment with 24,25-$(OH)_2D_3$ had no effect on PKC activity. When these cells were treated for the same period of time with analogue 3a, a dose-dependent increase in PKC activity was observed that was maximal at 10^{-7} M. Addition of either cycloheximide or actinomycin D to cultures treated with 10^{-9} to 10^{-7} 3a had no effect on the observed level of PKC activity. However, in cultures treated with 10^{-6} M 3a, both inhibitors caused a slight but significant decrease in PKC activity, although activity still was greater than in the vehicle-only cultures. In growth zone chondrocyte cultures treated with analogue 3b (Fig. 5A), PKC activity was increased in a comparable manner at all concentrations tested, and this increase was unaffected by either cycloheximide or actinomycin D.

Addition of 24,25-$(OH)_2D_3$ to resting zone chondrocyte cultures for 90 min significantly increased PKC activity, whereas 1,25-$(OH)_2D_3$ had no effect. When either analogue 3a (Fig. 4B) or 3b (Fig. 5B) was added to the cultures for the same period of time, no effect on PKC activity was observed, nor was there an effect of cycloheximide or actinomycin D.

The vitamin D metabolites and analogues 3a and 3b had direct effects on membrane PKC that were dose- and time-dependent. Moreover, the effects were cell maturation–specific. Treatment of matrix vesicles produced by growth zone cells with 1,25-$(OH)_2D_3$ for 9 min significantly inhibited PKC activity, while 24,25-$(OH)_2D_3$ was without effect (Fig. 6A). Analogue 3a but not 3b inhibited PKC activity in the matrix vesicles, but the degree of inhibition was less than that seen with 1,25-$(OH)_2D_3$ and occurred at a 100× higher concentration. PKC activity in plasma membranes from growth zone chondrocytes was also affected by hormone and analogue treatment (Fig. 6B). 1,25-$(OH)_2D_3$ stimulated PKC, whereas 24,25-$(OH)_2D_3$ and 3b were without effect. Analogue 3a, however, caused a dose-dependent increase in plasma membrane PKC, although the effect was significantly less than that produced by 1,25-$(OH)_2D_3$. Similar results were obtained when matrix vesicles and plasma membranes from growth zone cul-

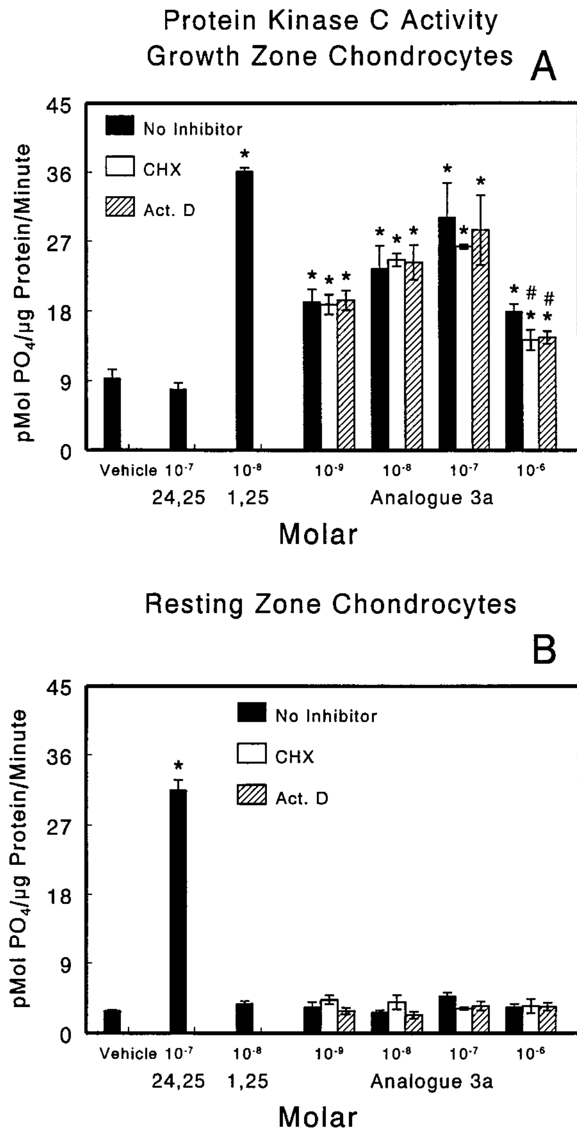


Fig. 4. Effect of vitamin D analogue 3a on PKC specific activity of growth zone and resting zone chondrocytes. Confluent, fourth passage growth zone (**A**) and resting zone (**B**) chondrocytes were treated for 9 and 90 min, respectively, with vitamin D vehicle (control), 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or 3a and PKC specific activity in the cell layer determined. Some cultures were treated with 0.1 mM cycloheximide (CHX), an inhibitor of translation, or 0.01 mM actinomycin D (Act. D), an inhibitor of transcription. The figure shows results from one of three identical experiments yielding similar results. Values represent the mean ± SEM for six separate cultures. $^{*}P < 0.05$, significantly different from vehicle-only control. $^{\#}P < 0.05$, significantly different from analogue treatment alone.

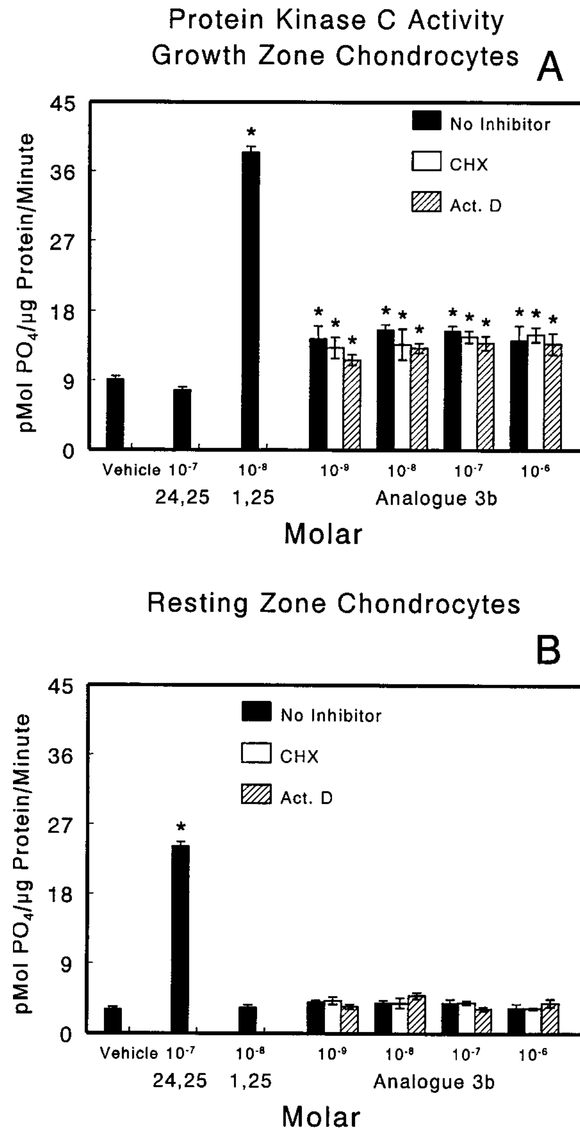
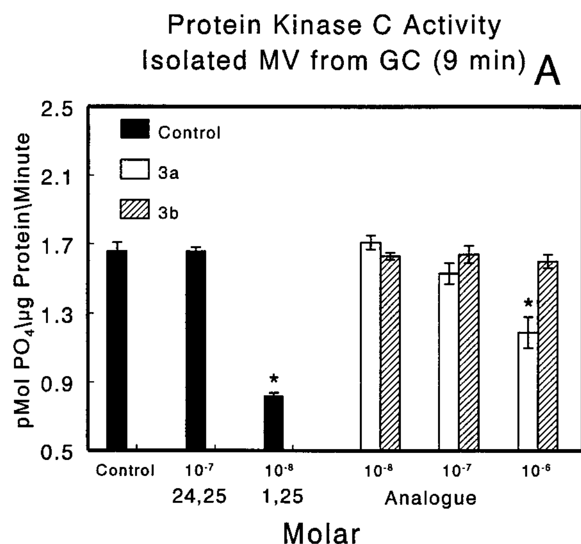
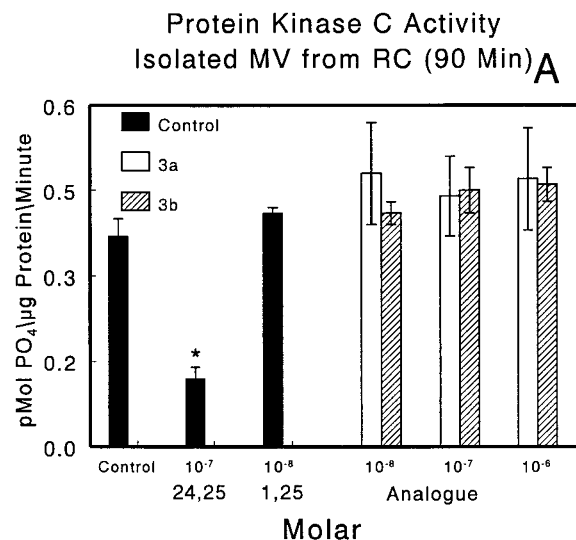


Fig. 5. Effect of vitamin D analogue 3b on PKC specific activity of growth zone and resting zone chondrocytes. Confluent, fourth passage growth zone (**A**) and resting zone (**B**) chondrocytes were treated for 9 and 90 min, respectively, with vitamin D vehicle (control), 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or analogue 3b and PKC specific activity in the cell layer determined. Some cultures were treated with 0.1 mM cycloheximide (CHX), an inhibitor of translation, or 0.01 mM actinomycin D (Act. D), an inhibitor of transcription. The figure shows the results from one of three identical experiments yielding similar results. Values represent the mean ± SEM for six separate cultures. $^{*}P < 0.05$, significantly different from vehicle-only control.

tures were incubated with the vitamin D metabolites or analogues for 90 min (data not shown).

PKC activity in matrix vesicles and plasma membranes from resting zone chondrocyte cultures was sensitive to the direct action of 24,25-$(OH)_2D_3$ and analogues 3a and 3b in a cell maturation–specific manner (Fig. 7). PKC activity in matrix vesicles was inhibited by treatment with 24,25-$(OH)_2D_3$ for 90 min, while 1,25-$(OH)_2D_3$ and analogues 3a and 3b were without effect (Fig. 7A). Plasma membrane enzyme activity was stimulated by treatment with





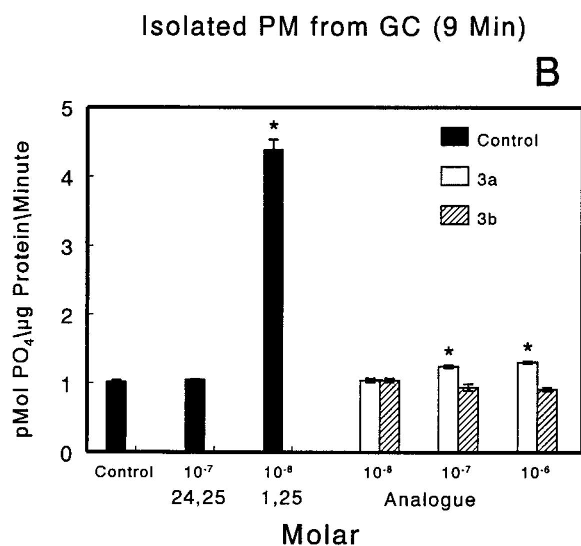


Fig. 6. The direct effect of vitamin D analogues 3a and 3b on PKC specific activity in matrix vesicles (MV) and plasma membranes (PM) isolated from growth zone chondrocyte (GC) cultures. MV (**A**) and PM (**B**) were isolated from fourth passage cultures and treated with vitamin D vehicle, 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or vitamin D analogue for 9 min and PKC specific activity determined. The figure shows the results from one of three identical experiments yielding similar results. Values represent the mean ± SEM for membranes from six separate cultures. $^*P < 0.05$, significantly different from untreated control.

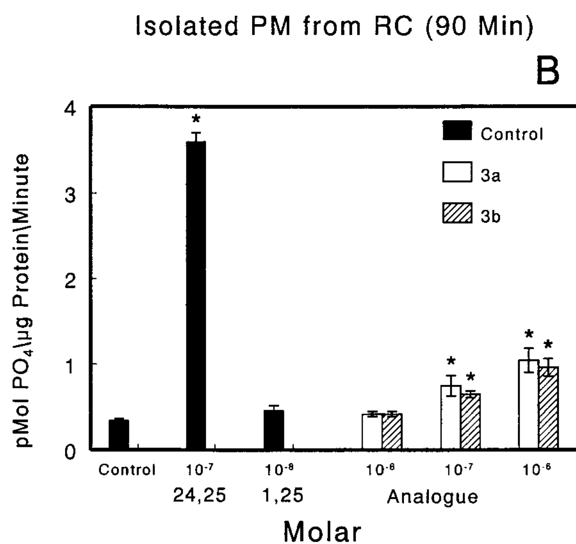


Fig. 7. The direct effect of vitamin D analogues 3a and 3b on PKC specific activity of matrix vesicles (MV) and plasma membranes (PM) isolated from resting zone chondrocyte (RC) cultures. MV (**A**) and PM (**B**) were isolated from fourth passage cultures and treated with vitamin D vehicle, 24,25-$(OH)_2D_3$ (24,25), 1,25-$(OH)_2D_3$ (1,25), or vitamin D analogue for 90 min and PKC specific activity determined. The figure shows the results from one of three identical experiments yielding similar results. Values represent the mean ± SEM for membranes from six separate cultures. $^*P < 0.05$, significantly different from untreated control.

24,25-$(OH)_2D_3$ for 90 min (Fig. 7B). Incubation with 3a or 3b increased PKC activity in a dose-dependent manner, but the effect was less potent than that observed with 24,25-$(OH)_2D_3$. After 9 min of exposure to the vitamin D metabolites or analogues, membrane PKC activity was affected in a similar manner to that seen at 90 min (data not shown).

DISCUSSION

The mechanisms by which the metabolites of vitamin D exert their effects on cells are complex. Part of the effect is mediated through the traditional vitamin D_3 receptor [Pike, 1992; Boyan et al., 1992]. The recent indications that membrane components are capable of binding

$1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$ [Nemere et al., 1994; Seo and Norman, 1996; Norman et al., 1994; Lieberherr et al., 1989] suggest that membrane receptors may play a role as well. Whether or not specific membrane receptors are involved in the mechanism of vitamin D_3 action, rapid membrane responses can elicit genomic effects via a number of signal transduction pathways including PKC. Alternatively, these rapid responses may be nongenomic, resulting in no new gene expression, again via a variety of signal transduction pathways.

In the present study, we examined the mechanism of vitamin D_3 action using specific hybrid analogues of $1,25\text{-}(OH)_2D_3$ which had very low binding capacities for the VDR [Posner et al., 1992, 1994, 1995; Posner and Dai, 1993] yet still had biological activity [Posner et al., 1992, 1993b]. Our results show that, despite the low VDR binding capacity, these hybrid analogues, like the active metabolites of vitamin D_3, regulated chondrocyte proliferation. This suggests that at least part of the antiproliferative effect is not through the traditional VDR pathway, although both genomic and nongenomic mechanisms may be involved.

The effects of the hybrid analogues on the chondrocytes varied, depending on the parameter being examined, implying that different aspects of the cellular response to vitamin D are mediated through independent mechanisms. While chondrocyte proliferation was inhibited by both analogues in both growth zone and resting zone cells, $[^{35}S]$-sulfate incorporation was regulated by both 3a and 3b in resting zone cells but only by 3b in growth zone cells. PKC activity was increased in growth zone cells by both analogues, but the dose dependence of the effect was analogue-dependent; neither analogue affected PKC activity in resting zone chondrocyte cultures.

There is an increasing body of evidence to support the hypothesis that gene expression in response to vitamin D metabolites can be regulated by signal transduction pathways other than the traditional VDR. Changes in intracellular Ca ion compartmentalization [Kim et al., 1994, 1996] as well as transcaltachia [Schwartz et al., 1991; Langston et al., 1990; Norman et al., 1992, 1994; Farach-Carson et al., 1991] may play a role. Our previous results support the contention that $1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$ mediate their effects, at least in part, through changes in PKC activity [Sylvia et al., 1993, 1996]. The observation that analogues 3a and 3b stimulate PKC activity in growth zone cells suggests that these compounds also operate via PKC in these cells. However, other mechanisms may be involved in the resting zone cells since increased PKC was not detected in response to the analogues.

It is likely that increased PKC activity is a rapid response to both 3a and 3b in both cell types. The effect in growth zone cells occurs by 9 min and is insensitive to either transcription or translation inhibitors, indicating that nongenomic mechanisms are involved. The mechanisms by which this occurs are not clear, however. At least for analogue 3b, direct effects on plasma membrane or matrix vesicle PKC do not appear to be involved. An alternative explanation is that 3b promotes the rapid translocation of existing cytosolic PKC to the membrane in the intact cell [Abou-Samra et al., 1989]. In contrast, analogue 3a does exert small but significant direct effects on plasma membrane PKC. This difference in the mode of action of 3a and 3b points to the stereospecificity of the mechanism.

In resting zone cells at 90 min, the time of maximum response to $24,25\text{-}(OH)_2D_3$, no effect of either 3a or 3b on cellular PKC is evident. Previously, we showed that the $24,25\text{-}(OH)_2D_3$–dependent increase in PKC involved genomic mechanisms [Sylvia et al., 1993], and we cannot rule this out for 3a or 3b if an increase in PKC expression did occur at some time earlier than 90 min. Recent studies indicate that PKC can be modulated directly through changes in phospholipid metabolism [Helm et al., 1996], which could account for the fact that isolated plasma membranes show dose-dependent increases in PKC activity when incubated directly with the analogues. Matrix vesicles isolated from resting zone cultures do not show a direct effect in response to either 3a or 3b, indicating that the elements needed for this response are not present. Several studies have shown that the composition of matrix vesicles produced by these cells is different from that of the plasma membrane as well as from matrix vesicles and plasma membranes isolated from growth zone chondrocyte cultures [Boyan et al., 1988a,b, 1992).

These experiments also show that, with respect to $[^{35}S]$-sulfate incorporation, 3b acted like, $1,25\text{-}(OH)_2D_3$ in growth zone cells and like $24,25\text{-}(OH)_2D_3$ in resting zone cells [Schwartz

et al., 1995]. This suggests that the active site on analogue 3b is common to the active sites on 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ as it pertains to this parameter and that cell specificity is conferred by the C-24 carbon. Previous studies demonstrate that neither singly modified analogue 2a nor 2b, which have the same A-ring modification as hybrid analogues 3a and 3b, affect [^{35}S]-sulfate incorporation in either cell type [Greising et al., 1996, in press], even though proteoglycan synthesis was sensitive to the vitamin D_3 metabolites, as reported previously [Schwartz et al., 1995]. This indicates that the A-ring may not play an active role in the interaction of the vitamin D metabolites with elements regulating glycosaminoglycan sulfation.

It may well be that the habit assumed by the vitamin D metabolites within the cellular and matrix vesicle membranes is due to charge density differences. However, it is also possible that the modification on the A-ring is such that it assumes a conformation like 24,25-$(OH)_2D_3$ when it interacts with membranes isolated from resting zone cell cultures. This is supported by the observation that analogue 3a behaved like 24,25-$(OH)_2D_3$ in both resting zone and growth zone cells cultures, causing a dose-dependent increase in [^{35}S]-sulfate incorporation in the former and having no effect in the latter.

The chemical structure of the analogue was important to its ability to regulate the chondrocytes. The cells were able to discriminate between stereoisomers. In the example presented above, 3a exerted an effect on [^{35}S]-sulfate incorporation in resting zone chondrocytes only, whereas 3b elicited a dose-dependent increase in both types of chondrocytes. While both 3a and 3b stimulated PKC in growth zone cells, dose dependence was demonstrated only for 3a. Moreover, neither metabolite elicited a detectable effect on PKC activity in the culture lysates. These data indicate that the stereochemical orientation of the A-ring hydroxymethyl group is important in regulating biological responses. In previous studies we showed that singly modified analogue 2a caused a marked increase in PKC activity, but its stereoisomer, analogue 2b, had only a minor effect on activity of this enzyme in growth zone cells and essentially no effect on PKC activity in resting zone cells [Greising et al., 1996, in press]. That this response is sensitive to minor variations in structure is evident by the difference in the magnitude of response elicited by hybrid analogues 3a and 3b in growth zone cells and the lack of response of either growth zone or resting zone chondrocytes to analogue 2b [Greising et al., 1996, in press]. Studies using isolated membranes demonstrate clearly the importance of the stereochemical orientation of the A-ring in the nongenomic response.

The A-ring modification, which is present in the type 2 singly modified analogues [Posner and Dai, 1993; Posner et al., 1992] and type 3 [Posner et al., 1994, 1995] hybrid analogues, is involved in the nongenomic activation of PKC. The type 3 hybrid analogues exerted their effects only on growth zone chondrocytes, whereas singly modified analogue 2a stimulated PKC activity in both types of chondrocytes. It is likely that the C,D-ring modification found in the type 3 hybrid analogues alters the conformation sufficiently to prevent the specific interaction needed to elicit the nongenomic response in the resting zone cells.

These results indicate that the membrane effects of the analogues are very specific and most probably mediated through receptors, although not the VDR. Only small changes in the structure of the analogues were sufficient to cause a major change in the biological response. The specificity conferred by the correct stereochemical configuration has been shown by others using 1α,25-$(OH)_2D_3$ vs. 1β,25-$(OH)_2D_3$ or 24R,25-$(OH)_2D_3$ vs. 24S,25-$(OH)_2D_3$ [Norman et al., 1992; Okamura et al., 1974; Baran et al., 1990]. This specificity of the nongenomic response is not limited to secosteroids but has also been demonstrated with steroid hormones as well. 17β- but not 17α-estradiol elicits rapid changes in membrane enzyme activity and fatty acid turnover in costochondral chondrocyte cultures and alters the fluidity of isolated membranes [Schwartz et al., 1996].

The biological role of 24,25-$(OH)_2D_3$ has been controversial for the last decade. Some studies suggest that it is only a weak metabolite [Norman et al., 1982], although there is mounting evidence that it plays an important role in cartilage differentiation [Yamaura et al., 1993; Nakamura et al., 1992; Lidor et al., 1987a; Ornoy et al., 1978; Schwartz et al., 1995] as well as in fracture healing [Lidor et al., 1987b,c]. This study confirms that a structural analogue of 1,25-$(OH)_2D_3$ had similar effects on chondrocytes as 24,25-$(OH)_2D_3$, suggesting that metabolites other than 1,25-$(OH)_2D_3$ play a role in

endochondral bone formation in general and on cartilage differentiation, specifically.

In summary, this study shows that selected biological responses can, in fact, be elicited by specific structural modification of vitamin D. Moreover, the results indicate that part of the effect of vitamin D metabolites on chondrocytes is not mediated through the VDR, and some of these effects are nongenomic. Finally, our findings suggest that specific membrane receptors for vitamin D metabolites are present in growth plate chondrocytes and they are regulated in a cell maturation–dependent manner.

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