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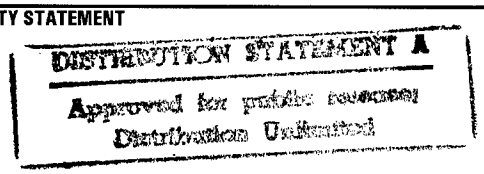

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A
THESIS

Presented to the Faculty of
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By
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San Antonio, Texas

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DEDICATION

With love, affection, and admiration, I dedicate this work, first and foremost, to my wife, Becky, and my daughter, Rachel. Their sacrifice, patience, and encouragement have helped to bring this project to fruition. I also dedicate this thesis to the spirit of my father, who shall always remain in my heart as my dad, my teacher, and my friend. Finally, I dedicate this work to my mother whose zest for life and learning continue to be an inspiration.

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It is with pleasure that I recognize Drs. Barbara Boyan and Zvi Schwartz for their commitment, not only to research and education, but truly to teaching. Being a teacher takes on a whole new meaning when compared simply to one who educates, and I am profoundly grateful for the guidance, energy, and insight Drs. Boyan and Schwartz have provided. I would also like to thank Drs. David Dean and Victor Sylvia whose dedicated efforts and understanding have helped bring this project to completion. I would like to recognize Monica Luna and the rest of the "Boyan Team," as it is difficult to imagine this project coming to completion without the laboratory expertise and attention to detail of this entire group. I am grateful to Dr. Gary Posner for providing the analogues utilized in this study and to Dr. Milan Uskokovich for the active metabolites utilized. In addition, I would like to applaud the Wilford Hall Department of Periodontics for their commitment to excellence in all endeavors.

VITAMIN D_3 ANALOGUES WITH LOW VITAMIN D RECEPTOR BINDING AFFINITY REGULATE CHONDROCYTE PROLIFERATION, PROTEOGLYCAN SYNTHESIS, AND PROTEIN KINASE C ACTIVITY

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The vitamin D metabolites, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, have a multiplicity of effects on chondrocytes. These effects are mediated through genomic action involving the classic vitamin D receptor (VDR) and various rapid membrane-mediated mechanisms that include both nongenomic and genomic effects. In intact cells, it is difficult to distinguish between genomic responses via the VDR and these other pathways. Recently, four potent analogues of 1,25-$(OH)_2D_3$ were synthesized: two that contain modifications in the A-ring only (2a, 2b), and two that contain modifications in both the A-ring and the side chain of the CD-ring (3a, 3b). Although these analogues are only 0.1% as effective in binding to the VDR as 1,25-$(OH)_2D_3$, they still retain many of the more therapeutically desirable properties of 1,25-$(OH)_2D_3$. Thus, they provide a novel approach for beginning to distinguish between VDR- and non VDR-mediated effects of 1,25-$(OH)_2D_3$. In this study, a well characterized cell culture model using rat costochondral resting zone (RC) and growth zone (GC) chondrocytes was used to study the effect of these analogues on [^{3}H]-thymidine incorporation, proteoglycan synthesis ([^{35}S]-sulfate incorporation),

and activity of protein kinase C by the cells. Confluent cultures were treated with 10^{-8}M 1,25-$(OH)_2D_3$, 10^{-7}M 24,25-$(OH)_2D_3$, or the analogues at 10^{-9} to 10^{-6}M for 24 hours. All analogues inhibited [^{3}H]-thymidine incorporation of both RC and GCs, as did 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. Neither analogue 2a nor 2b had an effect on proteoglycan production in RC or GCs. Similarly, hybrid analogue 3a had no effect on proteoglycan production by GCs, but treatment with 10^{-7} - 10^{-8}M 3a caused an increase in proteoglycan production by the RCs. This effect was blocked by actinomycin D and cycloheximide, inhibitors of transcription and translation, indicating that the effect of 3a on the RC cells was dependent on new gene expression and protein production. Hybrid analogue 3b caused a significant increase in proteoglycan production by both RC and GC cultures at concentrations of 10^{-8}-10^{-6}M and 10^{-8}-10^{-7}M, respectively. This activity was inhibited by actinomycin D and cycloheximide. Analogue 2a caused a dose-dependent stimulation of PKC activity that was not inhibited by cycloheximide or actinomycin D in either RC or GC cells. 2b, on the other hand, had no effect on PKC activity in either cell type. Hybrid analogues 3a and 3b affected PKC activity in only GCs; the effect of 3b was much less than that of 3a. This study demonstrates that these analogues, with little or no binding to the vitamin D receptor, affect proliferation, proteoglycan production, and PKC activity of chondrocytes. The effects are analogue specific and cell maturation-dependent. These results are consistent with the concept that the biological activity of vitamin D metabolites is mediated, at least in part, through nongenomic mechanisms.

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I. INTRODUCTION

Endochondral ossification is responsible for long bone lengthening during development and bone repair. During this process, a developmental cascade of chondrocyte differentiation and maturation occurs which results in mineralization of the cartilage matrix and later recruitment of osteoblasts to form new bone. Vitamin D is an important regulator of this process and is involved in many specific stages including chondrocyte differentiation and maturation (Atkin et al., 1985; Binderman and Somjen, 1984).

A. Vitamin D and Endochondral Ossification

Vitamin D is essential for proper endochondral ossification, and growth plate cartilage has been shown to be particularly sensitive to the vitamin D metabolites, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. Calcification of the growth plate matrix begins in extracellular organelles known as matrix vesicles, which are produced by chondrocytes *in vivo* (Anderson 1969; Ali et al, 1970) and *in vitro* (Hsu and Anderson, 1970; Golub et al 1983; Glaser and Conrad, 1981; Boyan et al., 1988a). In vitamin D deficiency, bone matrix synthesis and cartilage growth are inhibited, linear growth is halted, and bone formation is stunted (Raisz and Kream, 1983a and b). Restoration of mineralization and bone formation can be obtained through appropriate treatment with the active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$. Infusion of calcium has also been shown to heal rickets (Weinstein et al., 1984; Holtrop et al., 1986) leading to the suggestion that the primary function of 1,25-$(OH)_2D_3$ is to maintain extracellular calcium ion concentration. 24,25-$(OH)_2D_3$ has also been suggested to play a role in bone formation (Canterbury et al., 1980; Lieberherr et al., 1979; Ornoy et al., 1978). Rachitic rats have been shown to be responsive to injection of 24,25-$(OH)_2D_3$ either systemically (Atkin et al., 1985)

or locally into the upper tibial growth plate (Lidor et al., 1987). The state of differentiation of bone and cartilage cells plays a role in response to vitamin D metabolites. In general, less mature resting zone chondrocytes (RC) respond primarily to 24,25-$(OH)_2D_3$ whereas more mature growth zone chondrocytes (GC) respond primarily to 1,25-$(OH)_2D_3$ (Boyan et al., 1988a, 1992; Schwartz et al., 1988, 1989, 1990). Maturation-dependent effects are demonstrated by differences in extracellular protein synthesis (Schwartz et al., 1989), cell proliferation (Schwartz et al., 1989), matrix vesicle and plasma membrane enzyme activity (Schwartz et al., 1988a, b), arachidonic acid turnover (Schwartz et al., 1990; Swain et al., 1992), prostaglandin production (Schwartz et al., 1993), calcium flux (Langston et al., 1990), and vitamin D metabolite production (Schwartz et al., 1992a). 1,25-$(OH)_2D_3$ has been shown to directly change the function of osteoblasts and to change osteoclastic activity by some undefined osteoblast-osteoclast signal (Wong et al., 1977; Holtrop and Raisz, 1979; McSheehy and Chambers, 1987; Sato et al., 1991). Osteoclasts are also recruited by 1,25-$(OH)_2D_3$ from the monocyte-macrophage lineage of cells (Huh et al., 1987; Kurihara et al., 1989) but the site of action is not directly upon the osteoclasts. The recruitment of osteoclasts may explain why toxic levels of vitamin D result in hypercalcemia rather than hyperostosis.

B. Genomic Regulatory Mechanisms

Receptors for both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have been identified in cartilage (Corvol et al., 1980; Balmain et al., 1993; Fine et al., 1985). This suggests that many of the effects of these metabolites are mediated through the classical vitamin D receptor and involve changes in gene transcription or messenger RNA stabilization (Kyeyune-Nyombi et al., 1991; Gerstenfeld et al., 1990; Schwartz et al., 1989; Sylvia et al., 1993) and potentially other genomic regulatory mechanisms. Specifically, 1,25-$(OH)_2D_3$ has been demonstrated to affect

the synthesis of alkaline phosphatase and osteocalcin in chick limb bud mesenchymal cells (Boskey et al., 1992), chick sternal chondrocytes (Gerstenfeld et al., 1990), and chick epiphyseal chondrocytes (Hale et al., 1986). The effect of 24,25-$(OH)_2D_3$ on various markers of chondrocytic differentiation strongly suggest that genomic regulatory mechanisms are also involved (Sylvia et al., 1993, 1996). Studies using inhibitors of gene transcription and translation support this theory (Sylvia et al., 1993).

C. Non-Genomic Regulatory Mechanisms

Both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ also have nongenomic effects on chondrocytes and regulate the activity of various membrane enzymes. A nongenomic action, by definition, does not involve either new gene transcription or protein synthesis. Some examples of the many nongenomic actions of vitamin D that have been observed include changes in calcium flux (Schwartz et al., 1991; Farach-Carson et al., 1991; Langston et al., 1990), fatty acid metabolism (Schwartz et al., 1990; Swain et al., 1992), membrane fluidity (Swain et al., 1993), activation of protein kinase C (Sylvia et al., 1993), and inhibition of matrix vesicle enzyme activity as well as stimulation of plasma membrane PKC activity (Sylvia et al., 1996). Other systems have also been used to demonstrate the non-genomic effects of 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ has been shown to stimulate the hydrolysis of membrane phosphoinositides in enterocytes (Lieberherr et al., 1989; Wali et al., 1990), keratinocytes (McLaughlin et al., 1987; Tang et al., 1987), osteoblasts (Oshima et al., 1987) and parathyroid cells (Bourdeau et al., 1990). Three possible mechanisms have been proposed by which vitamin D metabolites may interact with cellular and matrix vesicle membranes: 1) vitamin D metabolites diffuse through the membrane to interact with the classic vitamin D receptor on the inner surface of the membrane (Kim et al., 1994); 2) specific membrane receptors exist which are distinctly

different from the classical vitamin D receptor (Zhou et al., 1992; Norman et al., 1994); and 3) a specific protein receptor does not exist and alteration of membrane fluidity results in changes in activity of membrane bound enzymes (Sheetz, 1993). Current evidence tends to support the mechanism of membrane receptors which are different than the classical vitamin D receptor.

D. Rat Costochondral Chondrocyte Model

Over the last several years, our laboratory has used cultures of rat costochondral chondrocytes to understand the differentiation and regulation of chondrocytes in the endochondral pathway (Boyan et al., 1992; Schwartz et al., 1988a, 1988b). In this model, the linear geometry of the growth plate is utilized to isolate resting zone and growth zone cells. Resting zone cartilage is separated from adjacent bone and proliferative zone cartilage by sharp dissection, and the hypertrophic zone (growth zone) is similarly dissected from the proliferative zone and calcified cartilage. Cells from each zone are then cultured separately, allowing study of their cell maturation specific differences. While chondrocytes are known to lose expression of phenotypic markers during long-term culture and subculture (Benya and Shaffer, 1982), these cells retain their ability to synthesize cartilage-specific proteoglycan and type II collagen through four passages in culture (Schwartz et al., 1989). These cultures have proven to be excellent models for studying both genomic and nongenomic effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ (Boyan et al., 1988; Langston et al., 1990; Schwartz et al., 1989; Swain et al., 1992; Sylvia et al., 1993). Both resting zone and growth zone chondrocytes produce extracellular matrix vesicles in culture, and their phospholipid composition (Boyan et al., 1988b) and enzyme activity (Boyan et al., 1988a; Schwartz and Boyan, 1988) are dependent on the cell of origin. Each type of matrix vesicle also differs

from the plasma membrane of the cell from which it is derived (Boyan et al., 1994a). The many advantages of this culture system are significant. Examination of the response of cells to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is possible by incubating cultures with hormone and examining either the cell layer or isolated matrix vesicles and plasma membranes. Addition of translation and transcription inhibitors can help distinguish between those responses which are genomic versus those which are nongenomic. Further, isolated matrix vesicles and plasma membranes can be incubated with hormone *in vitro* to evaluate nongenomic responses.

E. Vitamin D Analogues

Recent work in vitamin D research has focused on developing structural analogues with selective activity in what are considered nonclassical target tissues. Some of the most significant findings have revealed that several analogues are capable of inhibiting keratinocyte and/or malignant cell growth and promoting cellular differentiation in the absence of hypercalcemia or hypercalciuria (Bikkle, 1992).

Posner et al. (1992) have observed that virtually all of the leading therapeutic analogues of vitamin D contain a 1α-hydroxyl group on the A-ring, like vitamin D, and suggested that this substituent is required for biological activity. Several of these vitamin D analogues, modified on the A-ring, have been shown to be potent inhibitors of murine keratinocyte growth, as is 1,25-vitamin D_3 (calcitriol). Further, in some cases, antiproliferative activity has been observed in the absence of binding to the vitamin D receptor, suggesting the potential for therapeutic uses of these analogues (Posner et al., 1993b and c). Posner et al. (1993a and b) have reported on the synthesis of vitamin D analogues and characterized some of their biological activities. Depending on the specific modification, structurally modified 1α,25-

dihydroxyvitamin D_3, the hormonally active metabolite, has demonstrated a strong resistance to breakdown in human leukemic cells and potent inhibition of proliferation of murine keratinocytes (Posner et al., 1993 a and b).

Bishop et al. (1994) have studied the effect of modifying various regions of the 1α,25-$(OH)_2D_3$ molecule on binding to the human vitamin D binding protein (DBP), the principal transporter of vitamin D secosteroids in the plasma compartment. The study included 71 analogues, and depending on the specific modification, both increases and decreases in binding affinity were noted. For example, a 3- to 16-fold increase in ligand binding resulted from deletion of the 1α-hydroxyl group (Bishop et al., 1994) and was consistent with earlier studies showing that 25-$(OH)D_3$ had the highest affinity for DBP (Bouillon et al., 1980; Mallon et al., 1980). Structural analogues were shown to affect binding affinity in analogue specific fashion.

F. Matrix Vesicles

Recent studies have shown that vitamin D metabolites exert their effects through two major mechanisms. In the traditional pathway, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ bind to a cytosolic vitamin D_3 receptor and are translocated to the target cell nucleus, where the ligand-receptor complex interacts with the genome to regulate transcription (Boyan et al., 1992). In addition to its action in the nucleus, 1,25-$(OH)_2D_3$ has also been shown to modulate mRNA stability (Kyeyune-Nyombi et al., 1991). Receptors for both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have been identified in cartilage (Corvol et al., 1980; Balmain et al., 1993; Fine et al., 1985), suggesting that production of new matrix vesicles is under genomic control. In addition, it is now recognized that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ can act directly on the target cell membrane, causing rapid changes in calcium flux (Farach-Carson et al., 1991; Langston et al., 1990; Zhou et

al., 1992), fatty acid metabolism (Rasmussen et al., 1982; Matsumoto et al., 1981; Swain et al., 1992; Schwartz et al., 1990; Schwartz et al., 1992b), phospholipid metabolism (Lieberherr et al., 1989; Wali et al., 1990; Tang et al., 1987; Bourdeau et al., 1990), membrane fluidity (Swain et al., 1993), and protein kinase C activity (Sylvia et al., 1993; Sylvia et al., 1996). While these rapid actions may ultimately result in genomic effects, studies using matrix vesicles demonstrate that at least some of the effects of the rapid actions of vitamin D metabolites are nongenomic (Swain et al., 1993; Sylvia et al., 1996; Schwartz et al., 1988). Matrix vesicles are extracellular organelles which contain no DNA or RNA and, therefore, no possibility of activity with the genome. Both resting zone and growth zone chondrocytes produce matrix vesicles in culture (Boyan et al., 1988b), and many of the effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ result in changes in matrix vesicle composition and enzyme activity (Boyan et al., 1988a; Schwartz et al., 1992; Schwartz et al., 1989; Dean et al., 1996; Schmitz et al, 1996).

The hypothesis that costochondral chondrocytes regulate matrix vesicles through nongenomic vitamin D-dependent mechanisms is supported by several observations. The synthesis and secretion of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is regulated by growth factors and hormones (Schwartz et al., 1992a), providing a method for fine-tuning the amount of each metabolite. In addition, studies in which isolated matrix vesicles are incubated directly with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ demonstrate that these organelles are directly regulated by the metabolites in a cell maturation-dependent manner (Swain et al., 1993; Sylvia et al., 1996; Schwartz et al., 1988a and c; Boyan et al., 1994b).

G. Protein Kinase C Activity

Studies examining the regulation of protein kinase C activity in chondrocytes support the hypothesis that both genomic and nongenomic pathways are involved in 1,25-$(OH)_2D_3$ and

24,25-$(OH)_2D_3$ action. 1,25-$(OH)_2D_3$ has a rapid effect on PKC activity in growth zone chondrocytes which does not require either new gene transcription or protein synthesis (Sylvia et al., 1993). By contrast, 24,25-$(OH)_2D_3$ stimulates PKC activity in resting zone cells, but the effect requires both mRNA production and protein synthesis. When plasma membranes or matrix vesicles are isolated from either resting zone or growth zone cultures and then incubated with their target cell-specific vitamin D metabolite, plasma membrane PKC is stimulated, matrix vesicle PKC is inhibited (Sylvia et al., 1996). PKCα is the responsive isoform in plasma membranes, while PKCζ is the responsive isoform in matrix vesicles, offering a partial explanation of how the metabolites can elicit one effect in the cell and another, or opposite, effect, in the matrix.

These data demonstrate that vitamin D metabolites can modulate enzymes like PKC through rapid nongenomic action on pre-existing proteins or through the production of a new enzyme or other regulatory factors via genomic action, the latter involving either the vitamin D receptor (VDR) or an alternative signal transduction pathway. Recent studies indicate that phospholipid metabolism (Swain et al., 1992; Schwartz et al., 1990), particularly vitamin D-dependent alterations in prostaglandin production (Schwartz et al., 1992b), play a role. Similarly, changes in calcium flux, like those observed in chondrocyte (Langston et al., 1990) and osteoblast (Farach-Carson et al., 1991) cultures, and intestinal epithelium (Zhou et al., 1992), can modulate the actions of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$.

H. Analogues 2a, 2b, 3a, 3b

The fact that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ can elicit distinct cell maturation-specific effects in chondrocyte cultures in the absence of gene transcription or protein synthesis implies that either specific membrane receptors for each metabolite exist, which are differentially

distributed between the two cell types, or that the structural differences between the two vitamin D metabolites result in a differential interaction with other membrane components. Four new analogues of 1,25-$(OH)_2D_3$, which have modifications in the A-ring (analogue 2a and its stereoisomer, 2b), as well as in both the A-ring and the C,D-ring side chain (hybrid analogue 3a and its stereoisomer, 3b) were utilized in this study (Posner et al., 1992, 1993a, b, a, 1994, 1995; Posner and Dai, 1993) (Figure 1). The structure-function relationships of these analogues have been probed to distinguish between genomic effects mediated through binding to the VDR and any nongenomic effects which occur in the absence of VDR binding. Although all of these analogues have less than 0.1% binding capacity to the VDR when compared to 1,25-$(OH)_2D_3$ (Posner et al., 1992; Posner et al., 1994), they exhibit very high antiproliferative activity (Schwartz et al., 1988a, c; Posner et al., 1992; Posner et al., 1994; Posner et al., 1993a, b). In addition, they are 0.1% as potent as calcitriol (1,25-vitamin D_3) in calbindin D_{28k} induction (Posner et al., 1992). These analogues also stimulate calcium channel-opening instantaneously, a nongenomic process in rat osteosarcoma cells (Posner et al., 1993a, b; Posner et al., 1994). Using these analogues, the present investigation evaluated some of the structural aspects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ that play a role in their nongenomic actions.

I. Purpose

The purpose of the study was to determine the genomic and nongenomic mechanism of action of various analogues of vitamin D on resting zone (RC) and growth zone (GC) rat costochondral chondrocyte differentiation and proliferation *in vitro*. This study is intended to provide insight into how modifications of the A-ring and other structural modifications of vitamin D molecule affect the biological activity of these secosteroids. The effect of analogue

treatment on [^{3}H]-thymidine incorporation and proteoglycan production by RC and GC, as well as on PKC activity in RC and GC cell layers was examined.

II. MATERIALS AND METHODS

A. Chondrocyte Cultures

The cell culture system used in this study has been described previously in detail by Boyan et al. (1988a, b). Ribcages were removed from 125g male Sprague-Dawley rats and placed in Dulbecco's modified Eagle's Medium (DMEM). The resting zone and growth zone cartilage were separated, the intervening and adherent tissue removed, and then incubated overnight at 37^0C in DMEM containing antibiotics in an atmosphere of 5% CO_2/95% air and 100% humidity. The DMEM was replaced by two 20-minute washes in Hank's Balanced Salt Solution (HBSS), followed by sequential incubations in 1% trypsin for 1 hour and 0.02% collagenase for 3 hours. After the enzymatic digestion was complete, the cells were separated by filtration, collected by centrifugation at 500 x *g* for 10 minutes, resuspended in DMEM, and placed into T-75 flasks at seeding densities of 10,000 cells/cm^2 for resting zone cells and 25,000 cells/cm^2 for growth zone cells. Incubation was conducted overnight in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml vitamin C with 5% CO_2/95% air and 100% humidity. The culture medium was replaced at the end of that time and at 72-hour intervals. At confluence, cells were subcultured at the same seeding densities as before and allowed to return to confluence. Third passage confluent cultures were subpassaged into 24-well microtiter plates and grown to confluence. In all experiments cells were subcultured in this manner, since previous studies have demonstrated that these cells retain their differential responsiveness to vitamin D metabolites and growth factors through this number of passages in culture (Boyan et al., 1988a, b, 1992; Schwartz et al., 1988a, b, c, 1989, 1990, 1992a, b; Schwartz and Boyan, 1988; Swain et al., 1993).

B. Vitamin D Analogues

The experimental analogues were obtained through generous contribution from Dr. Gary H. Posner (The Johns Hopkins University, Baltimore, Maryland). Four analogues were used for the studies described below. Analogues 1α-(hydroxymethyl)-3β-hydroxyvitamin D_3 (2a) and 1β-(hydroxymethyl)-3α-hydroxyvitamin D_3 (2b) (Figure 1) were synthesized in a convergent manner by combining enantiomerically pure C,D-ring ketone 12 with highly enantiomerically enriched A-ring phosphine oxides. These A-ring chirons were prepared starting from thermal (4 + 2) cycloaddition of 3-bromo-2-pyrone and acrolein (Posner et al., 1992; Posner et al., 1993b; Posner and Dai, 1993).

Analogues 1α-(hydroxymethyl)-3β-hydroxy-20-epi-22-oxa-26,27-dimethylvitamin D_3 (3a) and 1β-(hydroxymethyl)-3α-hydroxy-20-epi-22-oxa-26,27-dimethylvitamin D_3 (3b) were synthesized by convergent coupling of 1-hydroxymethyl A-ring phosphine oxide with modified C,D-ring ketones (Posner et al., 1992; Posner et al., 1993b; Posner and Dai, 1993; Posner et al., 1994).

C. Treatment of the Cultures with Vitamin D Metabolites and Analogues

For each experiment, confluent cultures of fourth passage chondrocytes were treated for various time periods with either control or experimental DMEM containing various concentrations of vitamin D_3 metabolites. Each vitamin D_3 analogue was tested at 10^{-6}-10^{-9} M, which includes physiological and pharmacological doses of the active metabolites of vitamin D_3 [1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ (a gift from Dr. Milan Uskokovich, Hoffman-LaRoche, Nutley, NJ)]. 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were used at 10^{-8}M and 10^{-7}M, respectively, as positive controls. Ethanol, used as the vehicle, was diluted at least 1:5000 v/v

with DMEM prior to adding to the culture medium to minimize any toxic effects. Ethanol at the same concentration was used as an internal control.

D. [H^3]-Thymidine Activity

To determine the effect of hormone on DNA synthesis by the chondrocytes, [^{3}H]-thymidine incorporation was performed as described previously (Schwartz et al., 1989). GC and RC were grown to confluence in 96 well plates. The cells were preincubated for 48 hours in DMEM containing 1% FBS to induce quiescence. The medium was then replaced with DMEM containing 1% FBS and hormone or vehicle at the appropriate concentration and the incubation continued for another 24 hours. 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M 24,25-$(OH)_2D_3$ were used as positive controls. 2 hours before harvest, 50µl [^{3}H]-thymidine in DMEM (2µCi/ml) were added. At harvest, the cell layers were washed twice with cold PBS and three times with cold 5% trichloroacetic acid (TCA), with the TCA remaining on the cells for 30 minutes at $^-4^0$C during the third wash. TCA-precipitable material was dissolved in 1% sodium dodecyl sulfate, and radioactivity measured by liquid scintillation spectroscopy.

E. [^{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), it has been shown that the amount of radiolabeled proteoglycan released by GC and RC into the medium was less than 15% of the total radiolabeled proteoglycan (media and cell layer) synthesized. Because more than 85% of the radiolabeled proteoglycan was in the cell layer, we only examined 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) with media containing 10% FBS,

antibiotics, and 50 μg/ml vitamin C. Twenty-four hours before harvest, fresh media containing vehicle alone or varying concentrations of analogue or hormones were added. Four hours prior to harvest, 50 μl DMEM containing 13 μCi/ml $^{35}SO_4$ 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 protein content was then determined on a small aliquot of this sample utilizing a macro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The total volume was then 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 amount of [^{35}S]-sulfate incorporated was determined by liquid scintillation spectrometry and calculated as DPM/mg protein in the cell layer.

F. Protein Kinase C Activity

Confluent cultures of fourth passage chondrocytes were treated for various time periods with either control or experimental DMEM containing various concentrations of vitamin D_3 analogue or metabolite. Previous experiments in our lab have determined that protein kinase C (PKC) activity in growth zone chondrocytes was increased after treatment with 1,25-$(OH)_2D_3$, but not 24,25-$(OH)_2D_3$, and maximal stimulation was observed after 9 minutes. In contrast, PKC activity in resting zone chondrocytes was affected by 24,25-$(OH)_2D_3$, but not 1,25-$(OH)_2D_3$, and maximal stimulation was observed after 90 minutes (Sylvia et al., 1993). Therefore, for the current studies, analogues and vitamin D_3 metabolites were added to growth zone chondrocytes for 9 minutes and to resting zone chondrocytes for 90 minutes. 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 minutes on ice.

Chondrocyte cell layer lysates containing equivalent amounts of protein were mixed for 20 minutes with a lipid preparation containing phorbol-12-myristate-13-acetate, phosphatidylserine, and Triton X-100 mixed micelles, which provide the necessary cofactors and conditions for optimal PKC activity (Bell et al., 1986). To this mixture, a high-affinity myelin basic protein peptide and ^{32}P-ATP (25 μCi/ml) was added to a final assay volume of 50 μl. Following a 10-minute incubation in a 30°C waterbath, 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 liquid scintillation counter. To verify whether the kinase activity affected by the analogues was restricted to PKC activity and not due to other protein kinases, a specific inhibitor peptide corresponding to amino acid residues 19-36 of the PKC pseudosubstrate region was added to the PKC reaction tubes at a final concentration of 3 μM (Yasuda et al., 1990).

To determine if new gene transcription or protein synthesis were involved in the activation of PKC, chondrocyte cultures were treated with vitamin D_3 analogues or metabolites in the presence of actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of translation. Growth zone chondrocytes were incubated with analogue and 0.01 or 0.1 mM actinomycin D or cycloheximide for 9 minutes. Resting zone chondrocytes were incubated with analogue and 0.01 or 0.1 mM actinomycin D or cycloheximide for 90 minutes. Following treatment with the inhibitors, the cells were washed with PBS and assayed for PKC activity as described above.

G. Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) of six cultures. Figures contain data from representative experiments, and all experiments were performed a minimum of three times. Differences were determined by ANOVA and the 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.

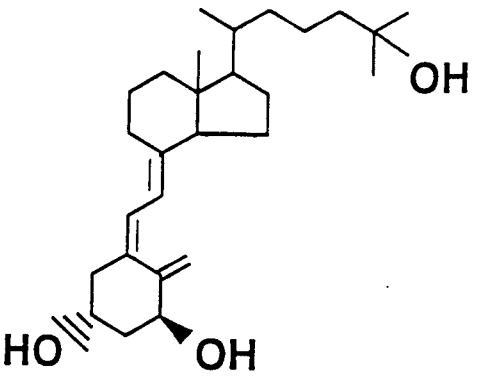
III. RESULTS

A. Cell Proliferation

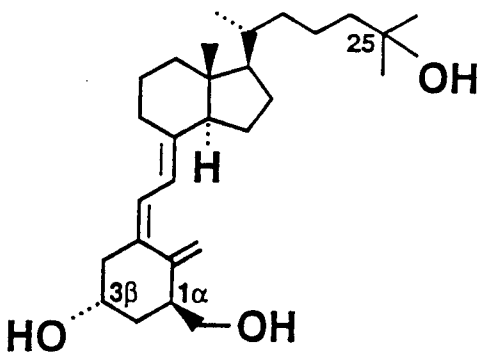
The addition of either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ to cultures of growth zone chondrocytes resulted in a significant decrease in [^{3}H]-thymidine incorporation by the cells (Figure 2A). The inhibition produced by 1,25-$(OH)_2D_3$ was greater than that produced by 24,25-$(OH)_2D_3$. When either analogue 2a or 2b was added to the cells, a dose-dependent inhibition of [^{3}H]-thymidine incorporation was produced (Figure 2A). The inhibition was less pronounced than that observed with 1,25-$(OH)_2D_3$ at the same concentration, but greater than that seen with 24,25-$(OH)_2D_3$. When hybrid analogue 3a or 3b was added to growth zone chondrocyte cultures, [^{3}H]-thymidine incorporation was also inhibited in a dose-dependent manner (Figure 3A). The degree of inhibition associated with analogues 3a and 3b was comparable to that caused by 24,25-$(OH)_2D_3$, but less than that caused by 1,25-$(OH)_2D_3$ at the same concentrations.

In contrast to the growth zone cells, [^{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 (Figure 2B). Addition of 2a to the resting zone cultures resulted in a dose-dependent inhibition of [^{3}H]-thymidine incorporation that was significant over the range of 10^{-8} to 10^{-6} M. The addition of 2b also inhibited [^{3}H]-thymidine incorporation by the cells, but only at higher concentrations (10^{-7} to 10^{-6} M). In addition, the inhibition with analogue 2b was markedly less than that seen with 2a or 1,25-$(OH)_2D_3$ (Figure 2B). The effect of hybrid analogues 3a or 3b on [^{3}H]-thymidine incorporation by resting zone chondrocytes was similar to that seen with 1,25-$(OH)_2D_3$, but the effect was only observed at the two highest concentrations tested (Figure 3B). Overall, each of

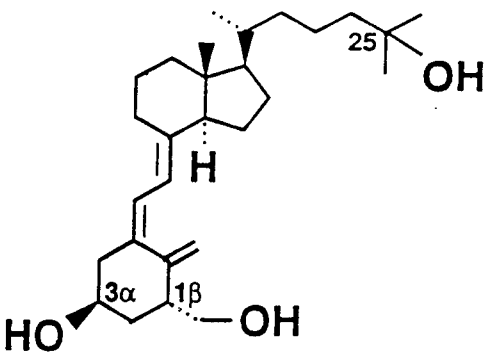
Figure 1. CHEMICAL STRUCTURES OF THE DIFFERENT VITAMIN D ANALOGUES USED IN THIS STUDY.



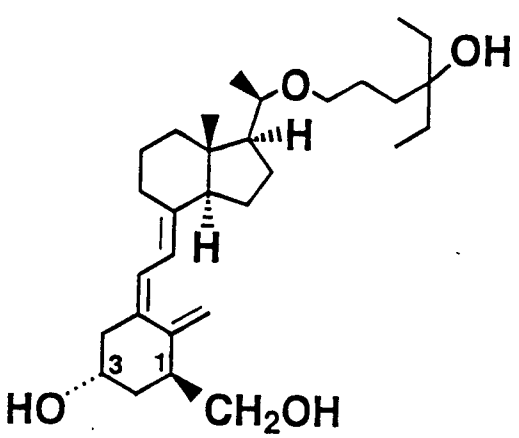
1α,25-(OH)$_2$ D$_3$



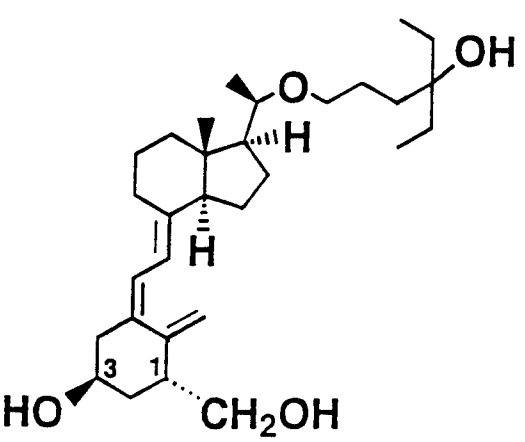
2a



2b



3a

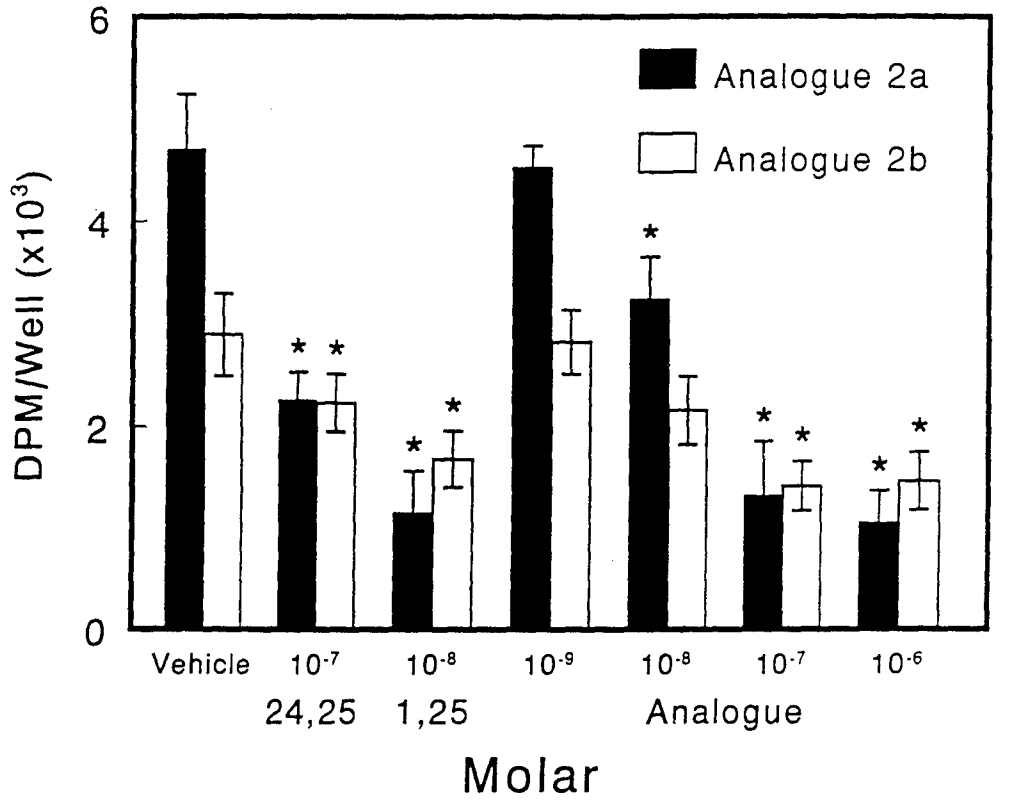


3b

Figure 2. THE EFFECT OF VITAMIN D ANALOGUES 2A AND 2B ON [3H]-THYMIDINE INCORPORATION BY GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (10-7 M) [24,25], 1,25-(OH)2D3 (10-8 M) [1,25], or 2a or 2b (10-9 to 10-6 M) and [3H]-thymidine incorporation determined. The figure shows the results of two separate experiments, one for 2a and one for 2b, 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.

Growth Zone Chondrocytes

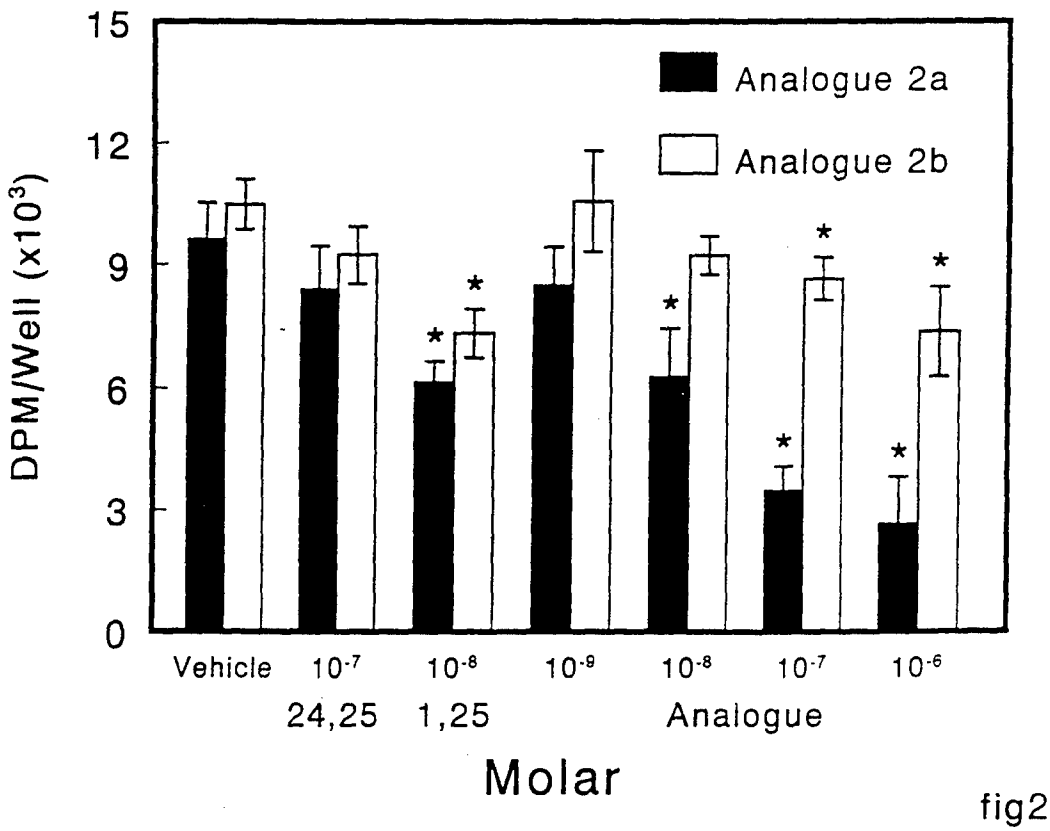
A



THyayb3

Resting Zone Chondrocytes

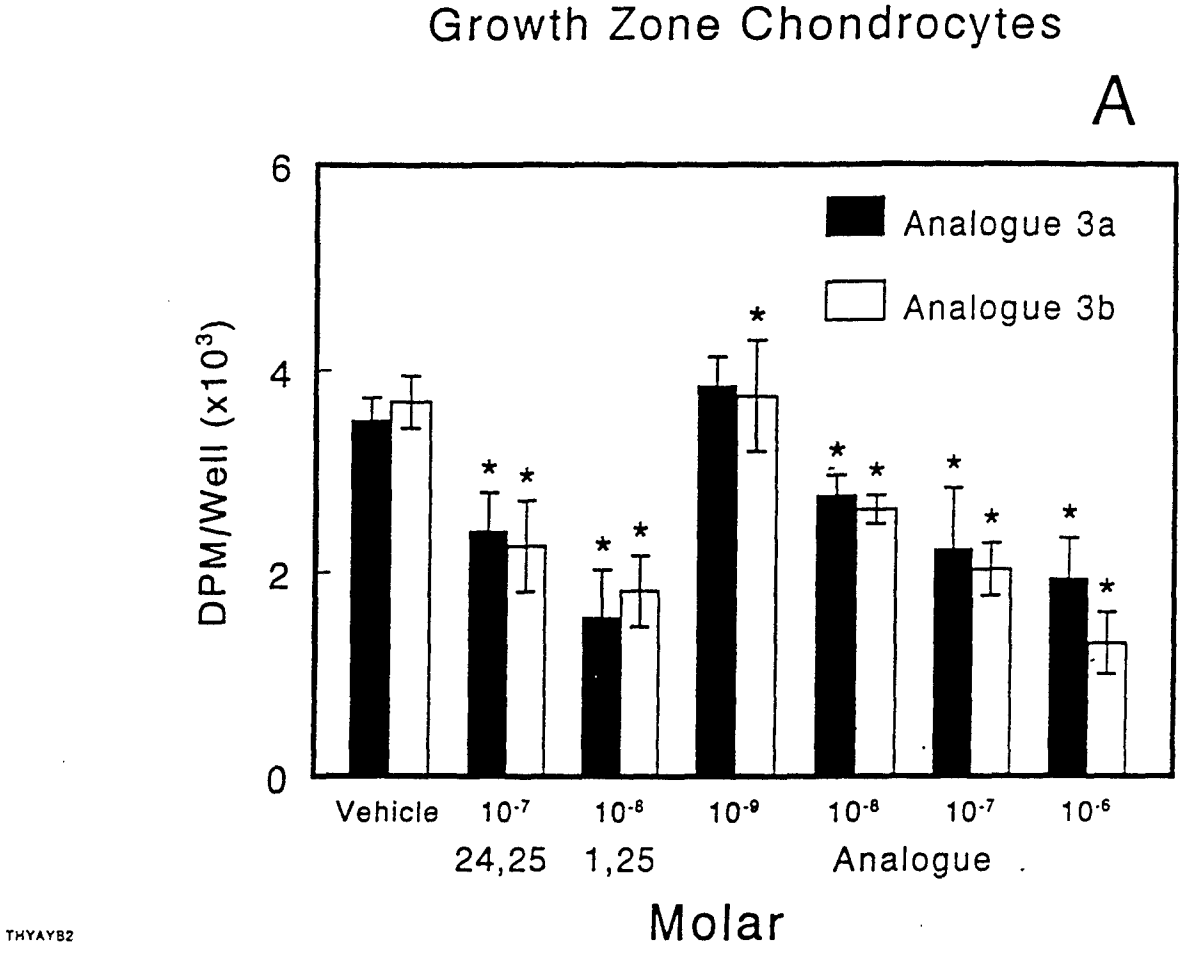
B



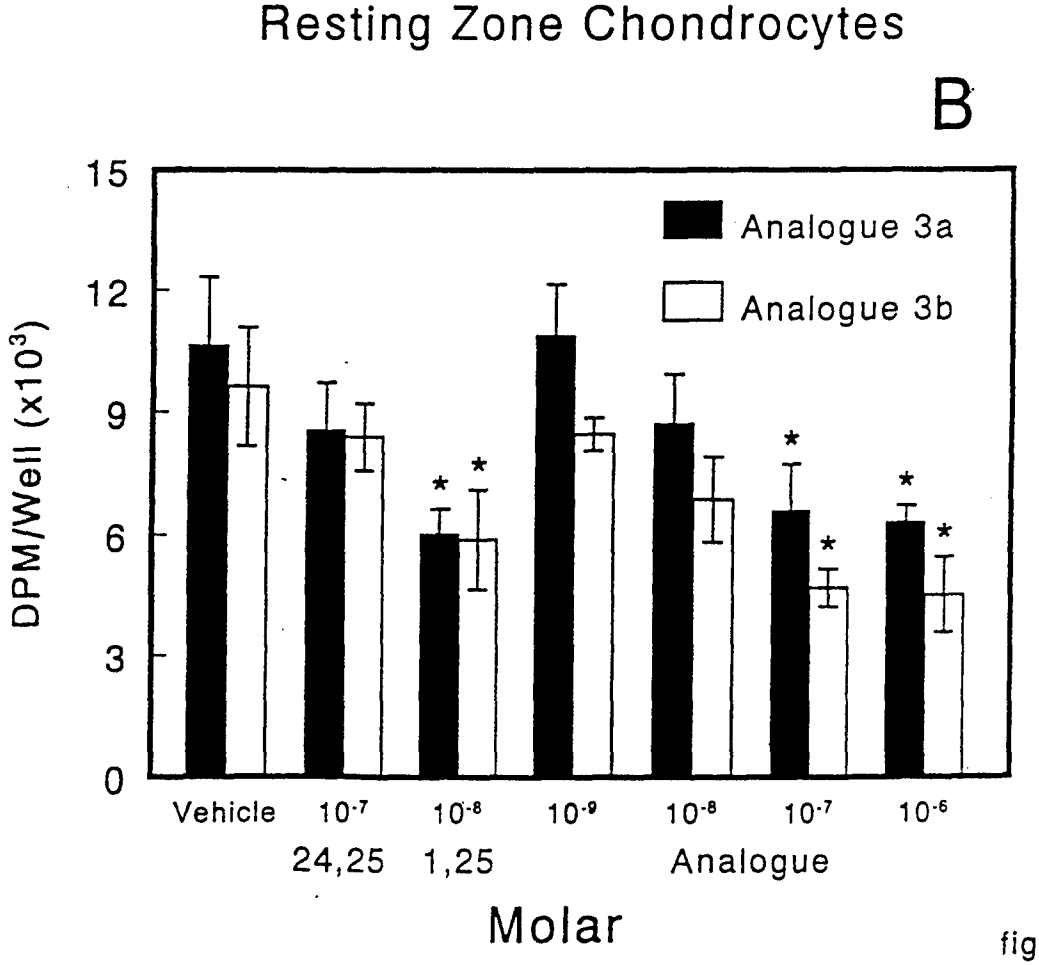
thya120

fig2

FIGURE 3. THE EFFECT OF VITAMIN D ANALOGUES 3A AND 3B ON [3H]-THYMIDINE INCORPORATION BY GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 3a or 3b (10-9 to 10-6 M) and [3H]-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.



THYAYB2



THYAYYB

fig3

the analogues inhibited cell proliferation with 2a, 3a, and 3b being similar in their potency of inhibition.

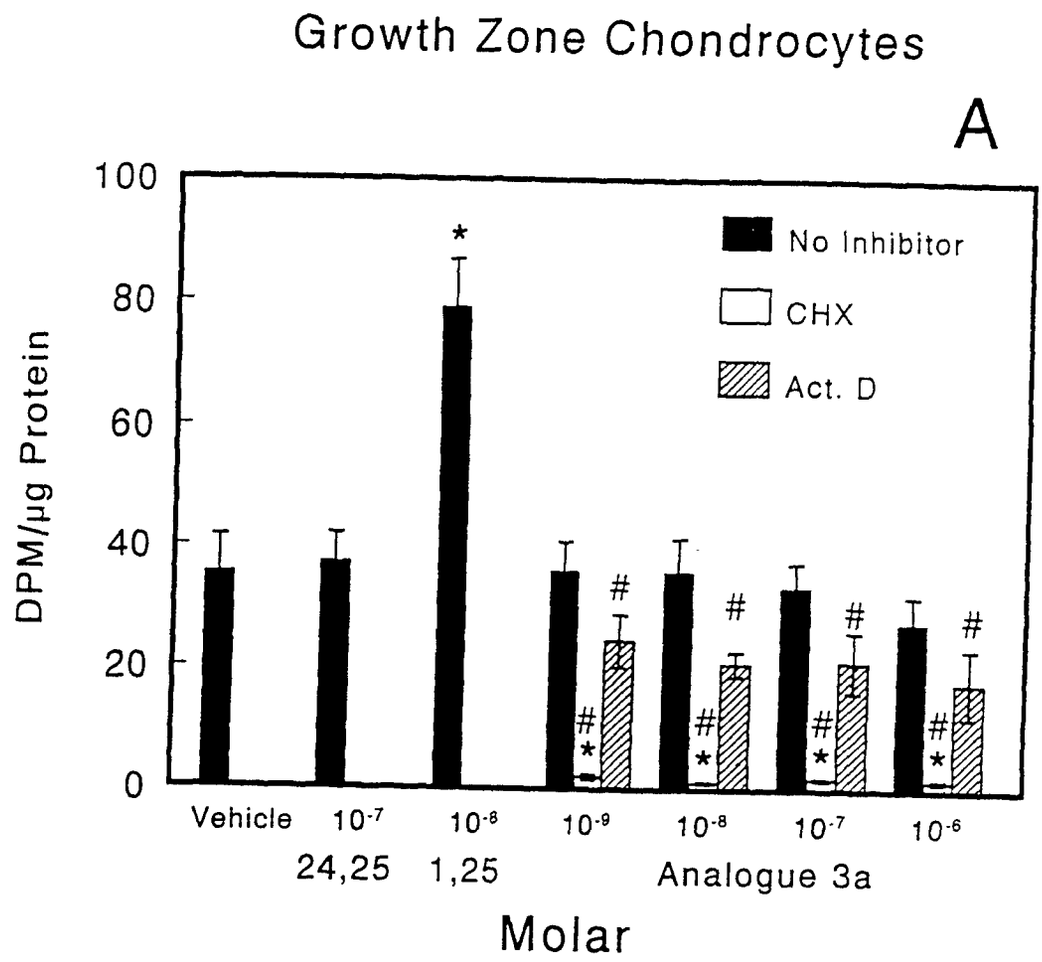
B. Proteoglycan Production

Analogues 2a and 2b had no effect on [^{35}S]-sulfate incorporation by either resting zone or growth zone chondrocytes (data not shown). When 1,25-$(OH)_2D_3$ (10^{-8} M) was added to cultures of growth zone chondrocytes, a significant increase in [^{35}S]-sulfate incorporation was observed (Figure 4A). In contrast, 24,25-$(OH)_2D_3$ was without effect. When the cultures were treated with analogue 3a, [^{35}S]-sulfate incorporation by the cells was unchanged. Addition of actinomycin D, an inhibitor of transcription, to the analogue-treated cultures, significantly reduced [^{35}S]-sulfate incorporation to less than the level seen in cultures without inhibitor. Addition of cycloheximide, an inhibitor of protein translation, reduced [^{35}S]-sulfate incorporation even more.

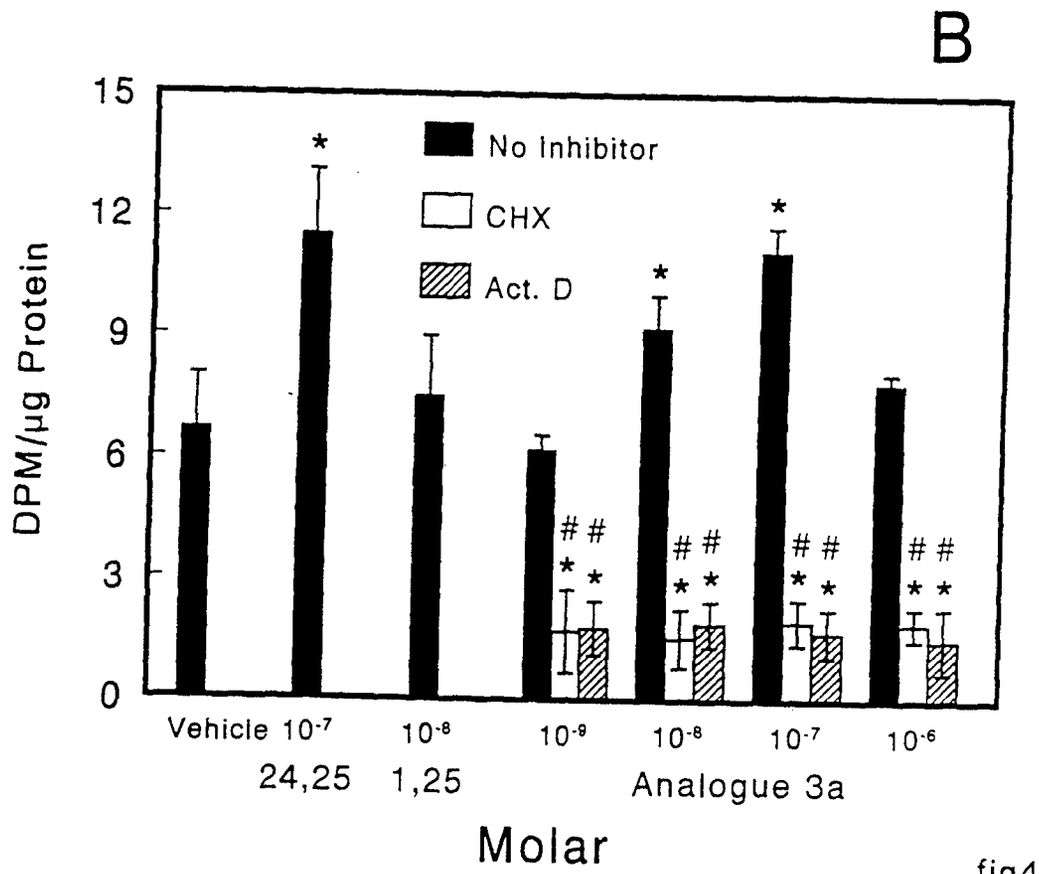
In contrast to the growth zone cells, [^{35}S]-sulfate incorporation by resting zone chondrocytes was significantly increased by treatment with 24,25-$(OH)_2D_3$, but *not* 1,25-$(OH)_2D_3$ (Figure 4B). Addition of the 3a hybrid analogue to the cultures produced a dose-dependent increase in [^{35}S]-sulfate incorporation, with a peak at 10^{-7} M, that was similar to the response of the cells to 24,25-$(OH)_2D_3$. Both cycloheximide and actinomycin D significantly inhibited [^{35}S]-sulfate incorporation by 3a-treated resting zone cells whether or not an effect of 3a was observed.

When the 3b hybrid analogue was added to either growth zone or resting zone chondrocytes, a dose-dependent stimulation in [^{35}S]-sulfate incorporation was observed (Figure 5) that was significant at analogue concentrations of 10^{-8} to 10^{-6} M (peak at 10^{-7} M) for growth zone cells and 10^{-8} to 10^{-7} M (peak at 10^{-8} M) for resting zone cells. The response to analogue 3b in GCs was similar to that of 1,25-$(OH)_2D_3$. The stimulatory effect of 3b was inhibited by

Figure 4. EFFECT OF VITAMIN D ANALOGUE 3A ON [35S]-SULFATE INCORPORATION BY GROWTH ZONE AND RESTING ZONE CHONDROCYTE CULTURES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 3a (10-9 to 10-6 M) and [35S]-sulfate incorporation 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 of one representative experiment, including positive 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. #$P < 0.05$ analogue alone v. analogue + inhibitor.



anproya3



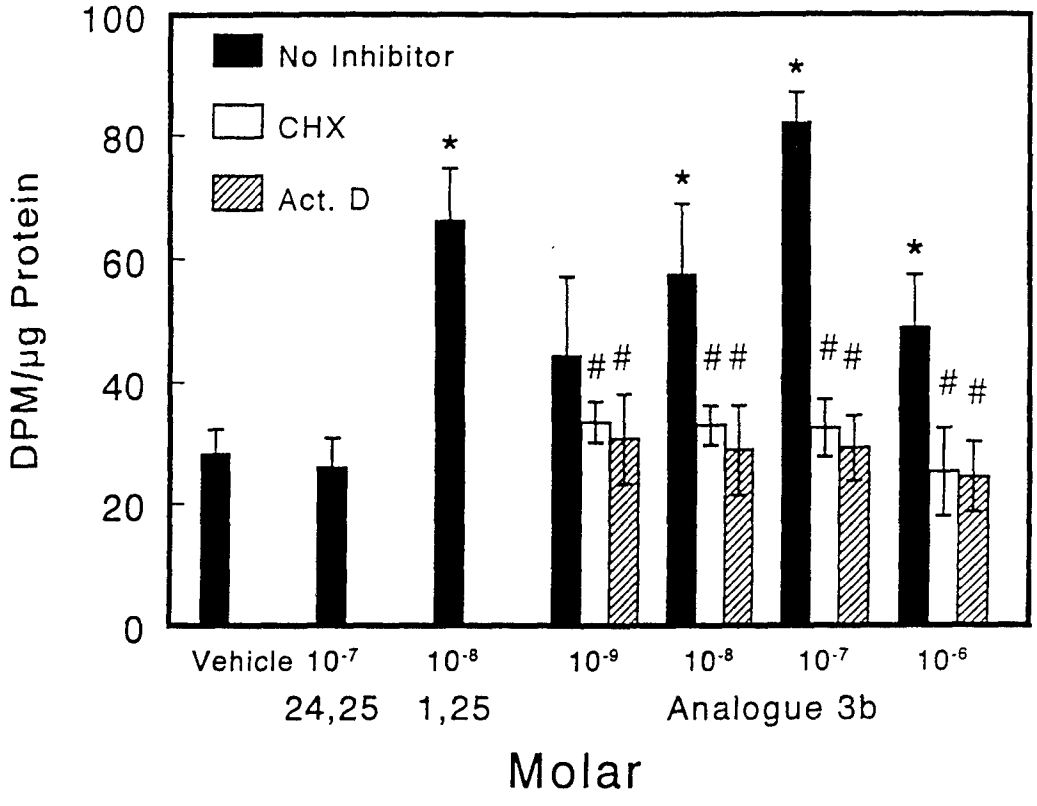
anproya

fig4

Figure 5. EFFECT OF VITAMIN D ANALOGUE 3B ON [35S]-SULFATE INCORPORATION BY GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 3b (10-9 to 10-6 M) and [35S]-sulfate incorporation 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 of one representative experiment, including positive 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. #$P < 0.05$, analogue alone v. analogue + inhibitor.

Growth Zone Chondrocytes

A



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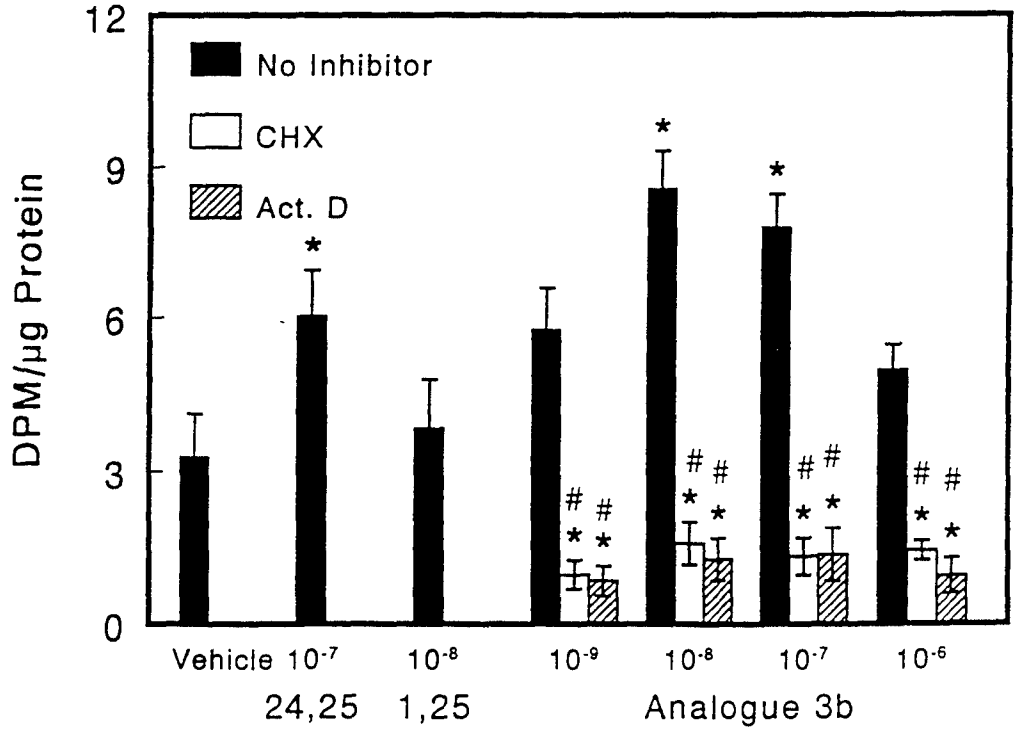
Resting Zone Chondrocytes

B



anproyb

fig5

cycloheximide and actinomycin D in both types of cells. Whereas in growth zone chondrocyte cultures, inhibition of mRNA and protein synthesis only reduced [^{35}S]-sulfate incorporation to control levels (Figure 5A), in the resting zone cell cultures, incorporation of radiolabel was reduced below control values (Figure 5B).

C. Protein Kinase C Activity

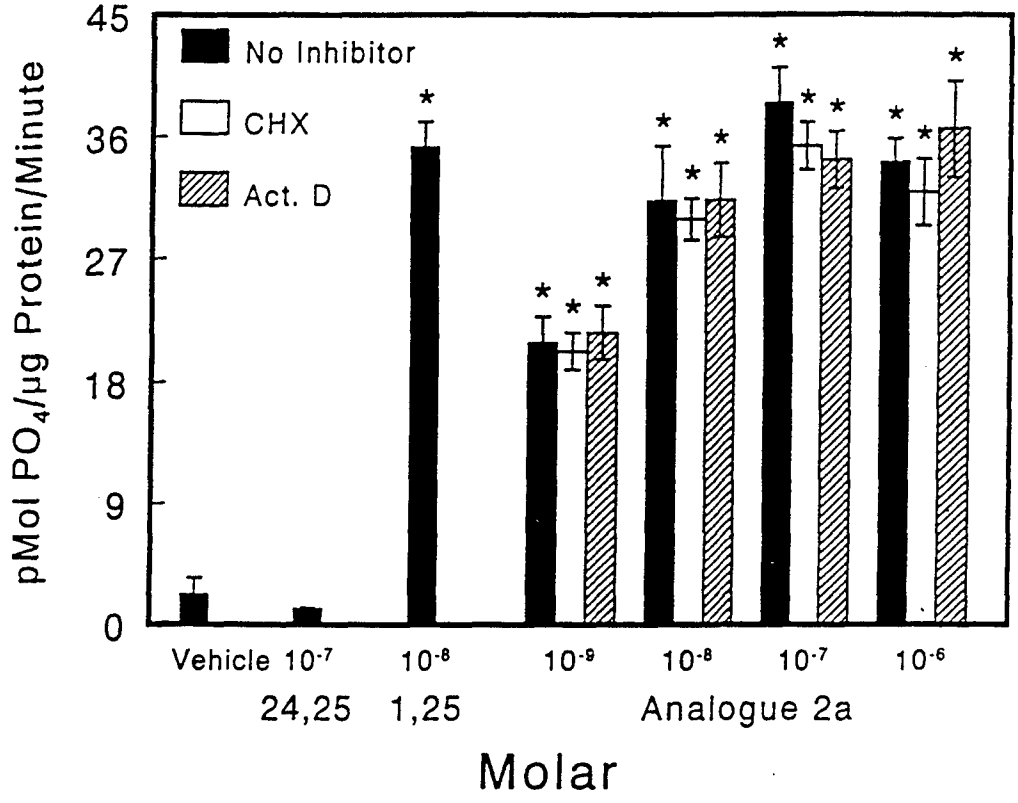
Treatment of growth zone chondrocytes with 1,25-$(OH)_2D_3$ produced a significant increase in PKC activity that was more than 30 times the level observed in untreated cultures (Figure 6A). In contrast, treatment with 24,25-$(OH)_2D_3$ had no effect on PKC activity. Treatment of growth zone cells with analogue 2a resulted in a dose-dependent increase in PKC activity that was maximal at 10^{-7} M. At concentrations of 10^{-8} M or higher, the effect of 2a was similar to the level found with 10^{-8} M 1,25-$(OH)_2D_3$ (Figure 6A). Addition of either translation or transcription inhibitors, cycloheximide or actinomycin D, to cultures treated with analogue 2a had no effect on the observed level of PKC activity.

Addition of 24,25-$(OH)_2D_3$ to resting zone chondrocytes significantly increased PKC activity, whereas 1,25-$(OH)_2D_3$ had no effect (Figure 6B). When analogue 2a was added to the cultures, there was a dose-dependent increase in PKC activity, that was maximal at 10^{-6} M. Analogue 2a, in concentrations from 10^{-7} to 10^{-6} M, resulted in enzyme activity comparable to that observed in cultures treated with 24,25-$(OH)_2D_3$. Analogue 2a was the only analogue to demonstrate an increase in PKC activity in RCs. Additionally, the level of enzyme specific activity measured was unaffected by addition of cycloheximide or actinomycin D to the culture (Figure 6B).

Figure 6. EFFECT OF VITAMIN D ANALOGUE 2A ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 2a (10-9 to 10-6 M) and protein kinase C 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 of one representative experiment, including positive 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 vehicle only control.

Growth Zone Chondrocytes

A



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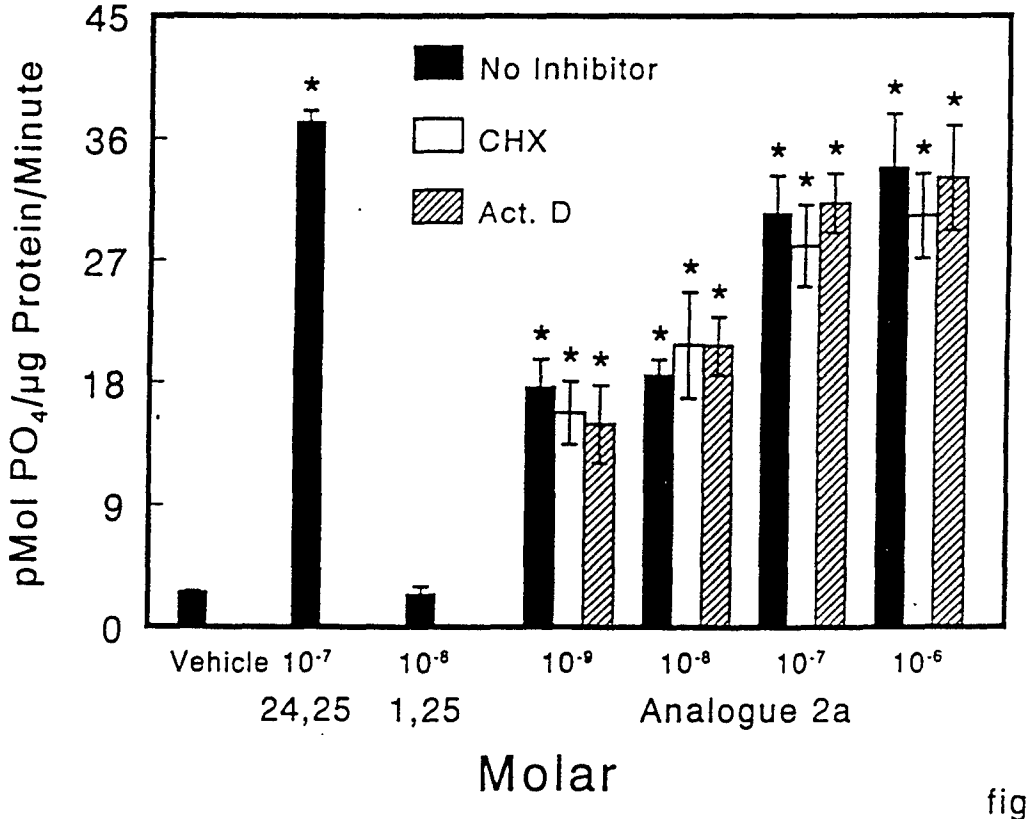
Resting Zone Chondrocytes

B



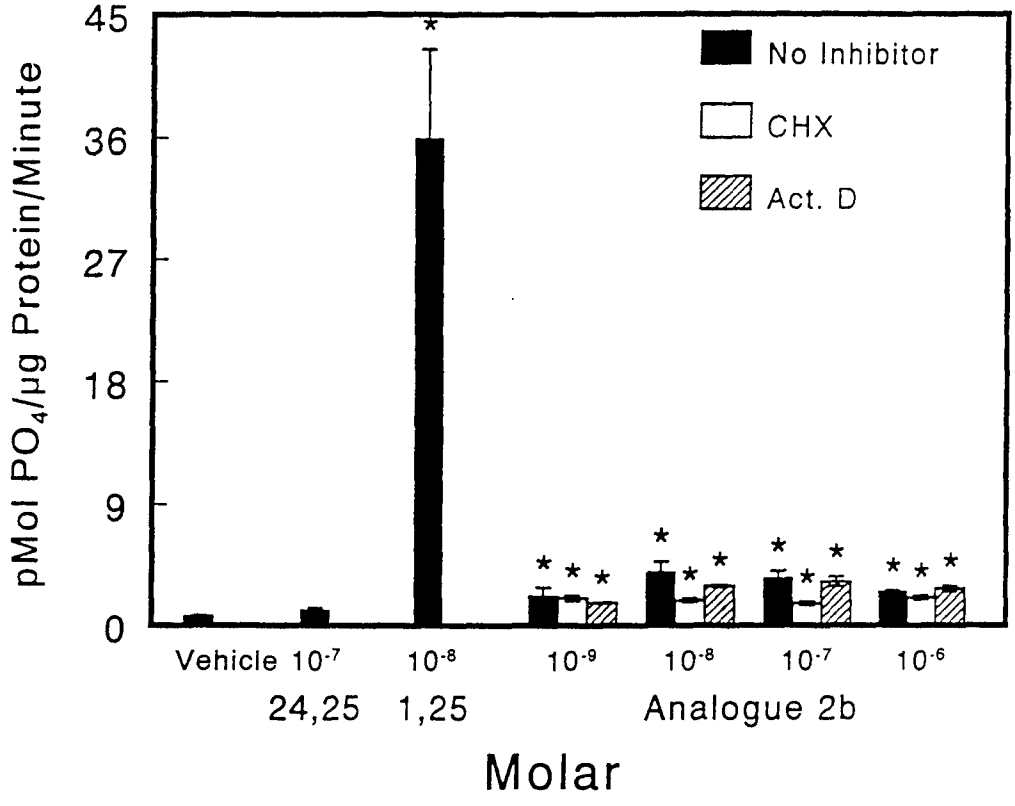
aNA1202

fig6

Figure 7. EFFECT OF VITAMIN D ANALOGUE 2B ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 2b (10-9 to 10-6 M) and protein kinase C 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 of one representative experiment, including positive 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 vehicle only control.

Growth Zone Chondrocytes

A



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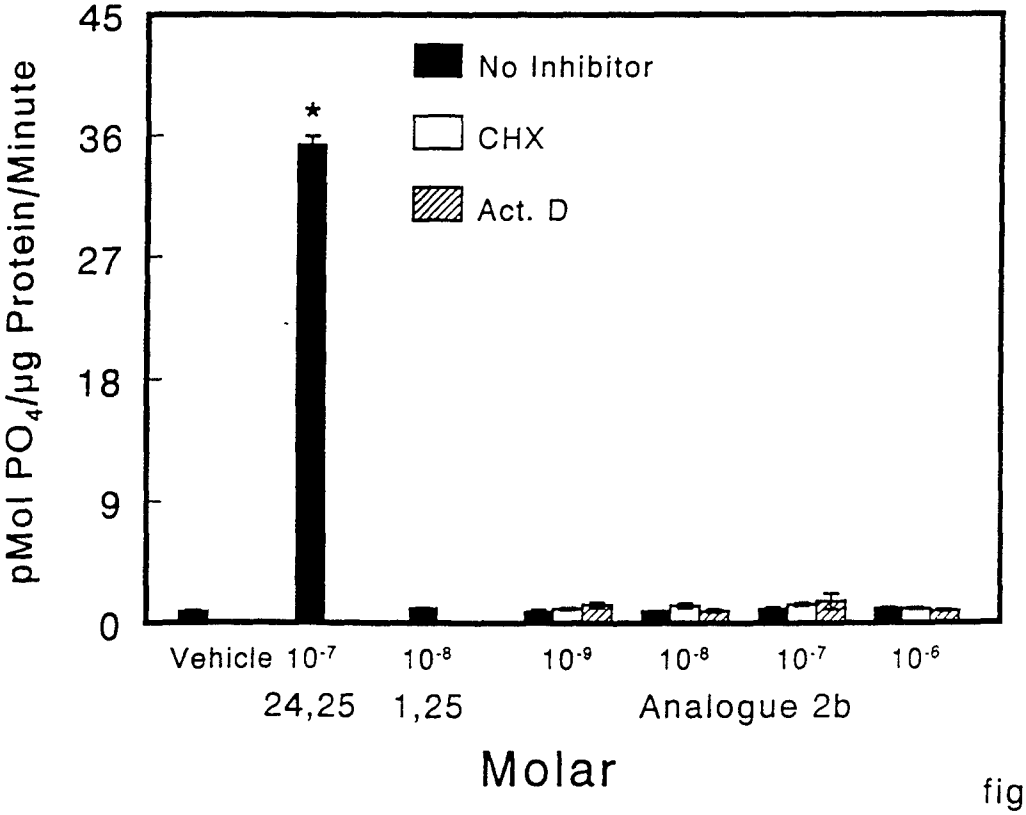
Resting Zone Chondrocytes

B



aNA1212

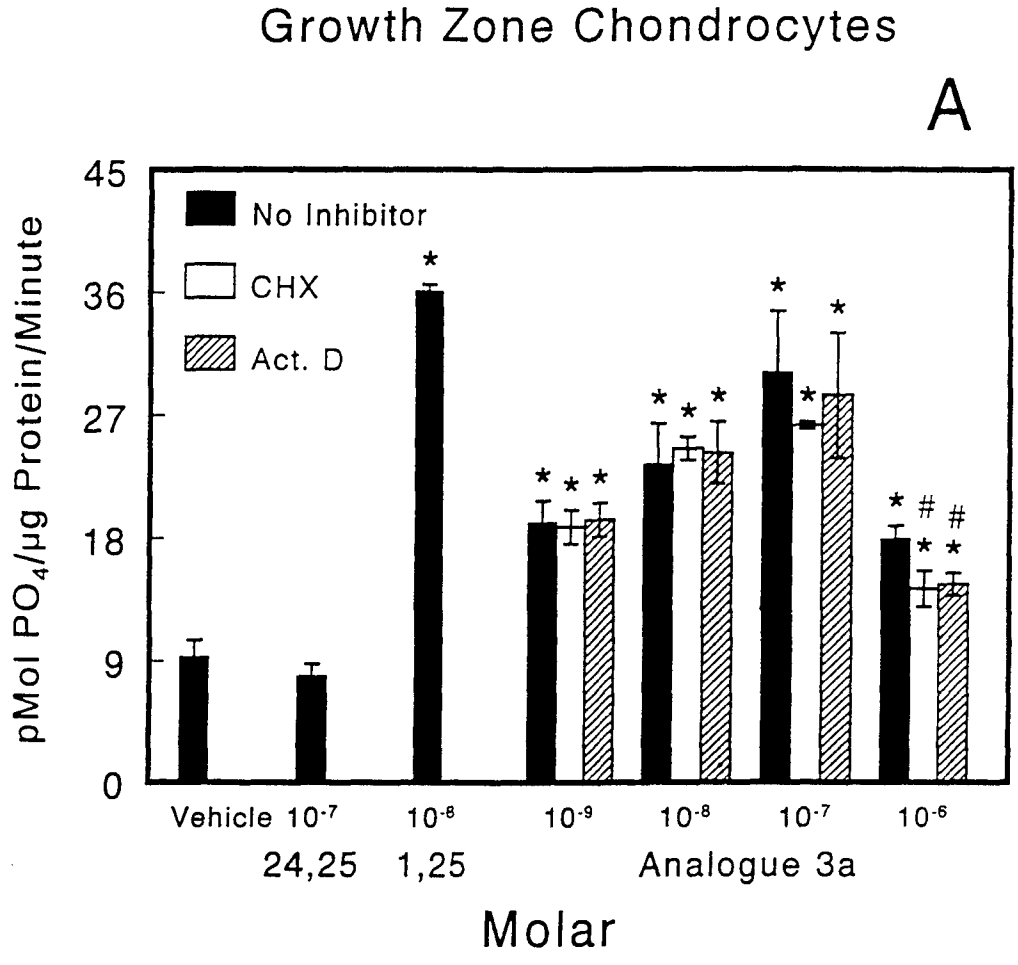
fig7

Analogue 2b caused a small, but significant, increase in PKC activity of growth zone chondrocytes at all of the concentrations examined (Figure 7A). This analogue had no effect on resting zone chondrocyte PKC activity, although 24,25-$(OH)_2D_3$ produced a significant increase (Figure 7B). Cycloheximide or actinomycin D treatment of either cell type had no effect on the observed level of PKC activity.

Hybrid analogue 3a had a dose-dependent, stimulatory effect on the PKC activity of growth zone chondrocytes that was maximal at a concentration of 10^{-7} M (Figure 8A). Only at this concentration was PKC activity comparable to that seen in cultures treated with 1,25-$(OH)_2D_3$. In cultures treated with 3a at 10^{-6} M, both cycloheximide and actinomycin D caused a partial inhibition in enzyme activity. In contrast, 3a had no affect on PKC activity in resting zone chondrocyte cultures (Figure 8B). There was no difference in the PKC activity of cultures treated with analogue alone or analogue with either cycloheximide or actinomycin D.

Hybrid analogue 3b stimulated PKC activity in growth zone cells (Figure 9A) in a comparable manner at all concentrations examined. This increase was unaffected by addition of either cycloheximide or actinomycin D. In contrast, 3b had no effect on PKC activity in resting zone cells, nor was there any effect of cycloheximide or actinomycin D (Figure 9B).

Figure 8. EFFECT OF VITAMIN D ANALOGUE 3A ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 3a (10-9 to 10-6 M) and protein kinase C 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 of one representative experiment, including positive 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 vehicle only control; #$P < 0.05$, analogue alone v. analogue + inhibitor.



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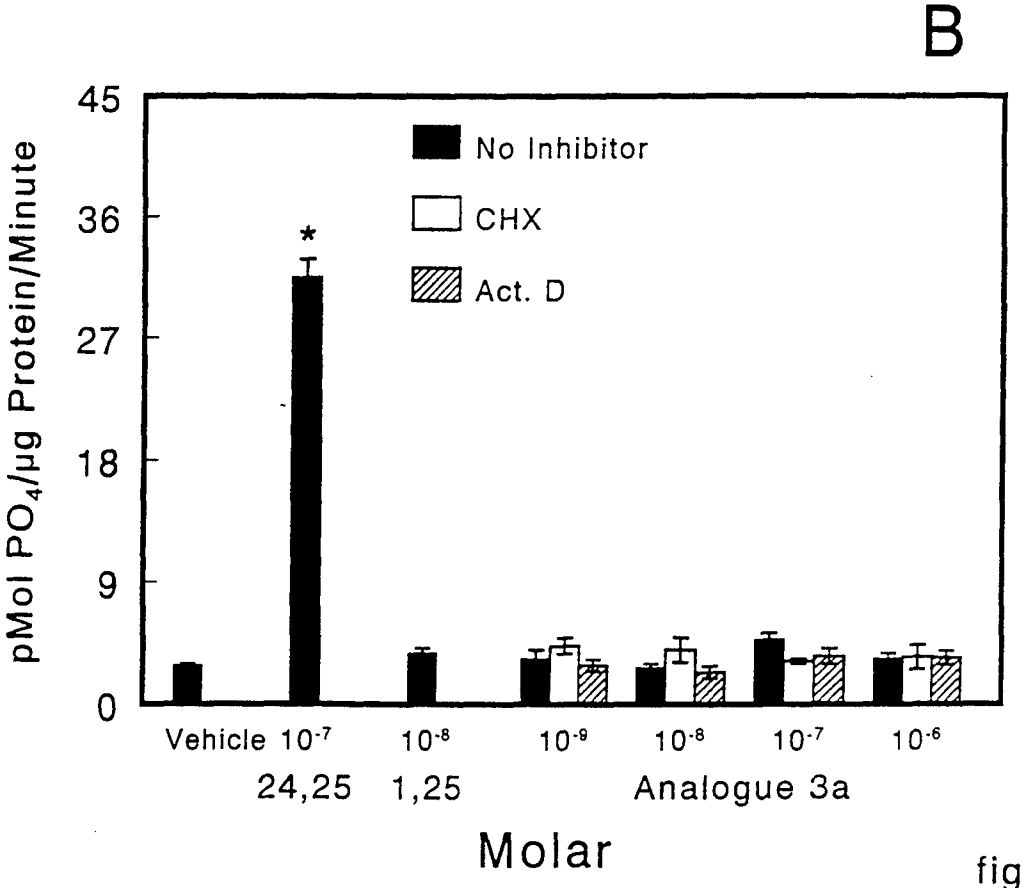

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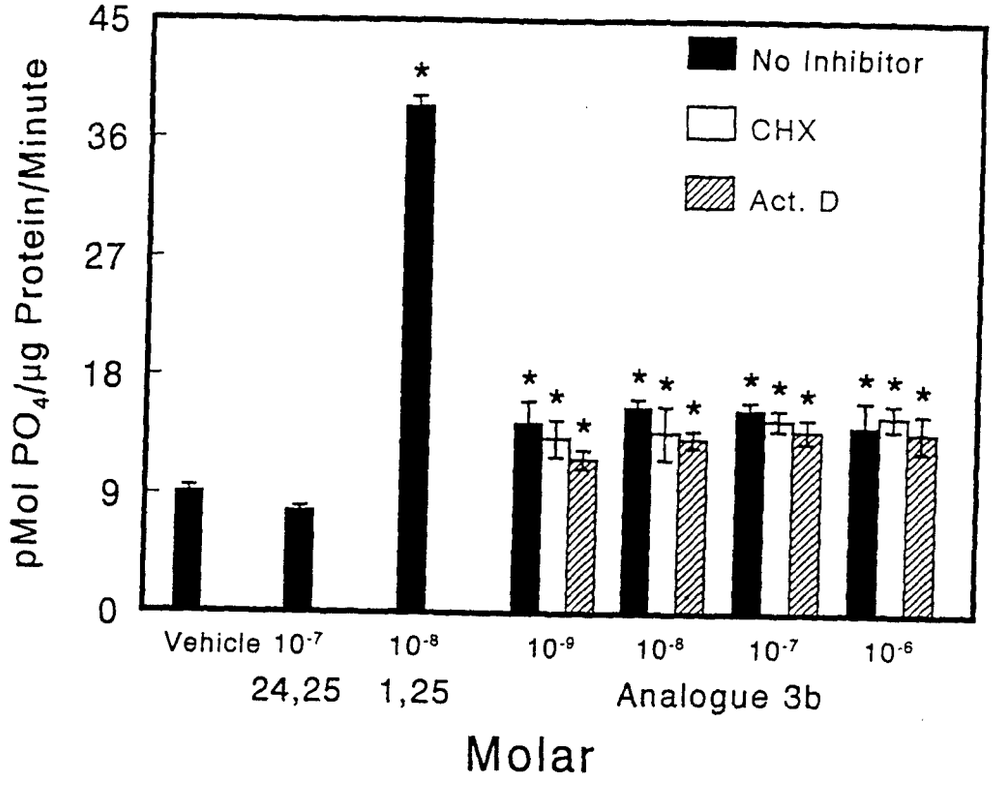
fig8

Figure 9. EFFECT OF VITAMIN D ANALOGUE 3B ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE AND RESTING ZONE CHONDROCYTES. Confluent, fourth passage growth zone (Panel A) and resting zone (Panel B) chondrocytes were treated for 24 hours with 24,25-(OH)2D3 (24,25), 1,25-(OH)2D3 (1,25), or 3b (10-9 to 10-6 M) and protein kinase C 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 of one representative experiment, including positive 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 vehicle only control.

Growth Zone Chondrocytes

A



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Resting Zone Chondrocytes

B

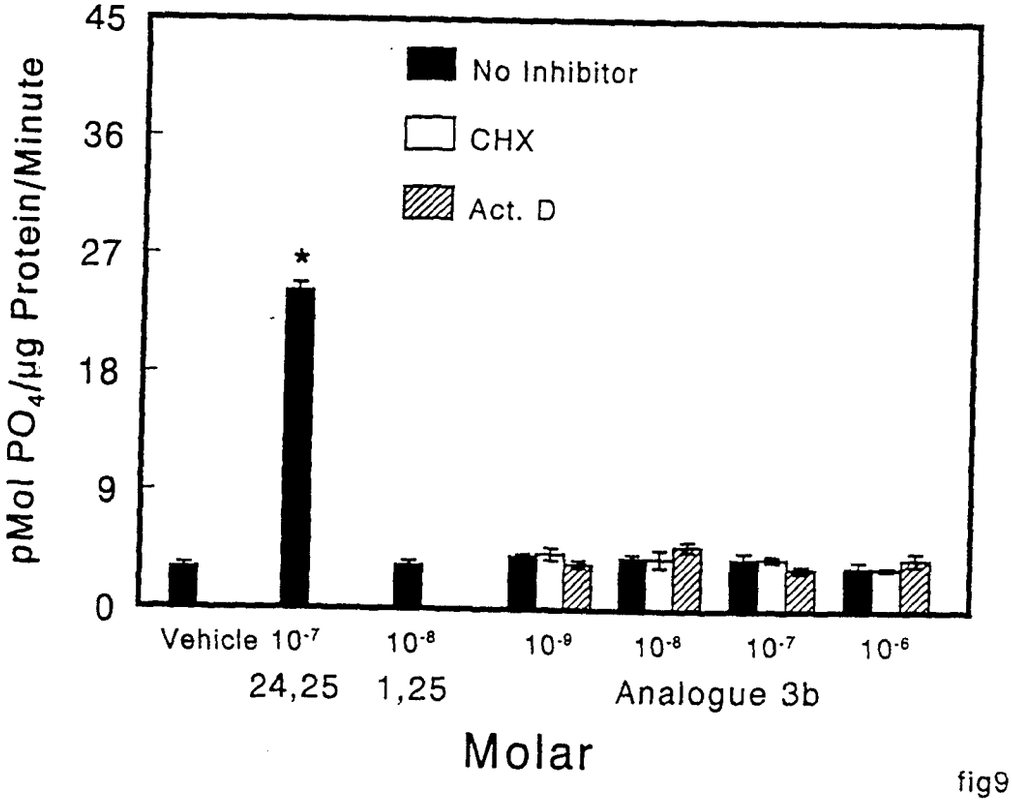

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fig9

IV. DISCUSSION AND SUMMARY

A. Vitamin D: A Historical Perspective

DeLuca (1979) presented a fine discussion on vitamin D metabolism and function, and the following historical points of interest are summarized from his work. The relationship between exposure to sunlight and skeletal structure was described in ancient times when the skulls of war victims were studied. While the Egyptians shaved their heads and wore limited clothing, the Persians prevented ultraviolet light from reaching their skin by wearing turbans and covering much of their body. As a result, the Egyptians had thicker, harder skulls than the Persians, and as we now know, this was due to increased circulating amounts of active vitamin D. Sir Edward Mellanby, early in the 1900's, produced a rickets-like disease in dogs that he was able to prevent or cure with cod liver oil. Since McCullom had found the fat-soluble vitamin A in cod liver oil, Mellanby concluded that vitamin A was able to prevent or cure rickets. McCullom pursued this finding further because of the differences in properties of the anti-rachitic substance and that of the growth promoting vitamin A; his experiments concluded that the ability to cure or prevent rickets was related to a new fat-soluble vitamin he called vitamin D. Steenbock demonstrated activation of provitamin D by ultraviolet light. Through further study, the identification of several D vitamins and the elimination of rickets as a major medical problem was achieved by the medical community. Schenck was able to produce vitamin D_3 by irradiation of 7-dehydrocholesterol in 1937. Some of the important aspects of the role of vitamin D in calcium metabolism were discovered by Nicolaysen; it was shown conclusively that vitamin D improves intestinal absorption of calcium and that vitamin D does not influence the endogenous loss of calcium or excretion into the intestinal tract. The

role of vitamin D in phosphorus metabolism by improving intestinal absorption of phosphate has been demonstrated by Harrison and Harrison. Carlsson showed vitamin D plays a role in the utilization of calcium from bone to support plasma calcium concentration, and this function was shown to be involved with the effects of parathyroid hormone by Harrison and also Rasmussen. (DeLuca, 1979). Since that time the interest in the role of vitamin D has continued. Vitamin D has been shown to be essential for proper endochondral ossification (Raisz and Kream, 1983a and b) as well as being an important regulator of chondrocyte differentiation and maturation (Atkin et al., 1985; Binderman and Somjen, 1984).

Since the late 1960's, researchers have begun to study the biological actions of vitamin D from the perspective that it was a steroid hormone with a nuclear receptor linked to the biological responses obtained instead of functioning as a vitamin (Norman, 1994). The hormonally active metabolite 1,25-$(OH)_2D_3$ has been shown to potently promote cell differentiation and inhibit cell proliferation (Tanaka et al., 1982; Abe et al., 1981). The potential therapeutic uses of vitamin D have been limited by the toxic effects of the active metabolites of vitamin D, primarily hypercalcemia (Berg et al., 1994; Elstner et al., 1994; Jung et al., 1994). It has been suggested that the potent bone-resorbing capacity of 1,25-$(OH)_2D_3$ *in vivo* and *in vitro* may be related to stimulation of marrow mononuclear cells to form osteoclasts (Roodman et al, 1985), and that excessive vitamin D is highly toxic because of ready access to cells (Ganong, 1987). Several synthetically derived analogues of vitamin D have been evaluated or used in clinical chemotherapy in such diverse conditions as osteoporosis, renal osteodystrophy, cancer, immunodeficiency syndromes, skin disorders, and diabetes (Reichel et al., 1989; Norman, 1994). These structural analogues of vitamin D are of

interest because of their therapeutic potential as well as their limited side effect profile potential.

B. Therapeutic implications

Evidence suggests that long-term calcium restriction and/or insufficient vitamin D may promote high blood pressure, colon cancer, and breast cancer in susceptible individuals in addition to bone fragility; conversely, improvement in calcium and/or vitamin D status may help alleviate some of these significant health concerns (Barger-Lux and Heaney, 1994). High doses of both dietary calcium and vitamin D have been associated with reduced development of precancerous changes in colonic mucosa, and preliminary findings suggest a protective effect against breast cancer for vitamin D (Barger-Lux and Heaney, 1994). The vitamin D analogue calcipotriol has been shown to raise serum calcium concentration and urinary calcium excretion 100-200 times less than 1,25-$(OH)_2D_3$ when administered intraperitoneally in rats (Colston et al., 1992a). Anti-tumor activity using the *in vivo* nitrosomethylurea-induced rat mammary tumor model was evaluated, and treatment with calcipotriol caused inhibition of tumor progression without the development of severe hypercalcemia. Whereas, treatment with 1 alpha$(OH)D_3$ produced similar inhibition of tumor progression but hypercalcemia developed (Colston et al., 1992a). Another vitamin D analogue, EB 1089, was shown to be at least an order of magnitude more potent than 1,25-$(OH)_2D_3$ at inhibition of the human breast cancer cell line MCF-7 proliferation *in vitro,* and *in vivo* evaluation showed EB 1089 inhibited rat mammary tumor growth in the absence of rising serum calcium levels while the same dose of 1,25-$(OH)_2D_3$ had no effect on tumor growth but caused hypercalcemia. Higher doses of EB1089 demonstrated striking tumor regression, although serum calcium levels did rise at the

therapeutic uses for vitamin D analogues as anti-tumor agents.

Another potential area of therapeutic significance that has been evaluated deals with leukemic cells. The $1,25(OH)_2$-20-epi-D_3 inhibited clonal growth of 87% of HL-60 myeloblast cells, 60% of S-LB1 cells (human T-cell lymphotropic virus type 1 [HTLV-1]-immortalized human T-lymphocyte cell line), and 50% of leukemic clonogenic cells from acute myelogenous leukemia patients (Elstner et al., 1994). The efficacy of all-trans retinoic acid in the treatment of acute promyelocytic leukemia is related to the ability of the retinoic acid to differentiate these peculiar leukemic cells, and studies have evaluated the potential for improvement of differentiation therapy by association of other differentiating agents with the retinoids. 1 alpha,25-dihydroxyvitamin D_3 was found to cooperate with the retinoids evaluated in inhibiting cell growth of the leukemic cell lines. Because of the effects of 1 alpha,25-dihydroxyvitamin D_3 on calcium metabolism limiting the therapeutic potential, the synthetic analogs MC903 and KH1060 were evaluated. Both agents were found to cooperate with retinoic acid, acting more efficiently than the natural molecule in inhibiting cell growth and inducing some aspects of cell differentiation (Defacque et al., 1994). The suggestion is made that vitamin D analogues may be useful in combination therapy with retinoids for the inhibition of leukemia cell growth with limited side effects.

The effects of various vitamin D analogues have also been evaluated in wound healing models. Intraperitoneal injections of vitamin D (cholecalciferol) have been shown to increase wound breaking strength and promote epithelialization significantly in dermal wound healing in Wistar rats (Ramesh et al., 1993). KH 1060, a 20-epi analogue of $1,25\text{-}(OH)_2D_3$ was evaluated in the normal and betamethasone-impaired granulation tissue formation in the polytetrafluoroethylene dead space model in hairless mice. Application of KH 1060 increased

polytetrafluoroethylene dead space model in hairless mice. Application of KH 1060 increased the indexes of fibroplasia and cellularity as well as collagen production and deposition of betamethasone-impaired granulation tissue; the effect of KH 1060 on normal connective tissue repair was less pronounced (Gniadecki and Serup, 1994). A study on the stimulatory effect of vitamin D on biochemical markers of bone remodeling in normal men suggested a synchronization and recruitment of new bone resorptive cells as well as marked increase in the serum level of osteocalcin, a biochemical marker of bone formative cells, with oral administration of 1,25-$(OH)_2D_3$ (Bollerslev et al., 1991).

Psoriasis is a chronic hyperproliferative skin disease in which both inflammatory and immunologic processes are suggested to play pathophysiologic roles (Kragballe, 1992a, b). 1,25-$(OH)_2D_3$ has been utilized in the treatment of psoriasis related to its inhibition of epidermal proliferation and promotion of epidermal differentiation (Kragballe, 1992a, b), however hypercalcemia and hypercalciuria are concerns (Berth-Jones and Hutchinson, 1992). Synthetic vitamin D_3 analogues have been developed and evaluated for the treatment of psoriasis vulgaris. Compared to 1,25-$(OH)_2D_3$, calcipotriol, the most extensively evaluated synthetic derivative in the treatment of psoriasis, has been shown to be about 200 times less potent in its effect on calcium metabolism yet apparently similar in receptor affinity, and topical calcipotriol has been shown to be efficacious for the treatment of plaque-type psoriasis (Kragballe, 1992a). These findings clearly demonstrate the therapeutic potential of these agents.

Another area of interest involves the relationship between the pancreatic β-cell and the vitamin D endocrine system. While few studies clearly link a nutritional vitamin D deficiency

D deficiency impairs insulin secretion in the perfused rat pancreas, and that administration of vitamin D_3 to the pancreatic islets can lead to normalization of glucose-stimulated insulin secretion (Norman et al., 1980; Chertow et al., 1983; Kadowaki and Norman, 1985; Billaudel et al., 1989).

Osteoporosis, a common problem which affects approximately 20 million Americans, primarily elderly women, is a disorder characterized by decreased bone mass and increased susceptibility to fractures (Rubin, 1991). An uncoupling of the bone resorption and bone formation sequence with an exaggeration of resorption, a reduction in formation, or a combination of both is generally associated with the cause of osteoporosis. In a comprehensive review, Rubin discusses two different types of osteoporosis, type I (postmenopausal) and type II (senile). The primary etiology of type I osteoporosis is thought to be associated with estrogen deficiency. Without estrogen, parathyroid hormone (PTH) causes an increase in bone turnover and mobilization of calcium from bone resulting in lower serum PTH which secondarily reduces 1 alpha-hydroxylation of 25-hydroxyvitamin D and impaired absorption of calcium from the gastrointestinal tract (Riggs and Melvin, 1986). The impaired ability of aging kidneys to synthesize 1,25-$(OH)_2D_3$ related to an age-related decrease in 1-alpha hydroxylase activity appears to be responsible for the development of type II osteoporosis (Tsai et al 1984). It is suggested that 1,25-$(OH)_2D_3$ may play a direct role in osteoblast function regulation because of the 1,25-$(OH)_2D_3$ receptors of osteoblasts (Rubin, 1991). Other contributing factors to type II osteoporosis include poor dietary intake of calcium (Heaney et al, 1978), inadequate dietary intake of vitamin D, inadequate sun exposure, and a decreased ability of the skin to produce vitamin D (MacLaughlin and Holick, 1985; Holick, 1986). While vitamin D has not been approved by the FDA for use in the

1985; Holick, 1986). While vitamin D has not been approved by the FDA for use in the treatment of osteoporosis, it is commonly used for this purpose (Rubin, 1991). Studies evaluating the therapeutic value of vitamin D supplementation in the treatment of osteoporosis have demonstrated the development of hypercalcemia (Aloia et al, 1988; Jensen et al, 1982).

C. Conclusions

Vitamin D metabolites exert their effects on cells through complex mechanisms. Part of the effect is mediated through the traditional vitamin D_3 receptor (Boyan et al., 1992). The recent identification of a membrane protein capable of binding 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ (Lieberherr et al., 1989; Corvol et al., 1980; Balmain et al., 1993; Fine et al., 1985; Norman et al., 1994), suggests that membrane receptors may play a role as well. Whether or not a specific membrane receptor is 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. It is also possible that these rapid responses may occur in the absence of new gene transcription, in a nongenomic fashion, again via a variety of signal transduction pathways.

In this study, the mechanism of action of vitamin D_3 was evaluated using specific analogues of 1,25-$(OH)_2D_3$ which had very low binding capacities for the VDR (Posner et al., 1992, 1994), yet still had biological effects (Schwartz et al., 1988a; Posner et al., 1992, 1993a, b, 1994). Compared to 1,25-$(OH)_2D_3$, these analogues have less than 0.1% binding capacity to the VDR, yet they maintain high antiproliferative activity (Schwartz et al., 1988a; Posner et al., 1992, 1993a, b, 1994). The results show that despite the low VDR binding capacity, these analogues, like active metabolites of vitamin D_3, inhibited chondrocyte proliferation, increased proteoglycan production, and increased PKC activity (which by itself can mediate the effects of the analogues) (Sylvia et al., 1993; Sylvia et al., 1996; Schwartz et al., 1995). This suggests that, although both

metabolites on the chondrocytes is not through the traditional VDR pathway. The increase in sulfate incorporation in response to hybrid analogue 3b was inhibited by both cycloheximide and actinomycin D, inhibitors of translation and transcription, indicating that new mRNA production and protein synthesis are necessary. In contrast, stimulation of PKC activity by analogue 2a was unaffected by either inhibitor, indicating that pre-existing enzyme was affected by treatment. The nongenomic regulation of PKC has been further supported by showing that PKC activity is modulated by the analogues in isolated matrix vesicles (data not shown).

The chemical structure of the analogue was important in its ability to regulate the chondrocytes. The cells were able to discriminate between stereoisomers. For example, hybrid analogue 3a had no effect on proteoglycan production in growth zone chondrocyte cultures whereas analogue 3b caused a marked dose dependent stimulation in [^{35}S]-sulfate incorporation. Similarly, 2a caused a marked increase in PKC activity in growth zone cells, comparable to that seen in the presence of 1,25-$(OH)_2D_3$; in contrast, its stereoisomer, analogue 2b, had only a minor effect on activity of this enzyme in these cells. These data indicate that the stereochemical orientation of the A ring hydroxymethyl group is important in regulating biological responses.

These results indicate that the effects of the analogues are very specific and most probably are mediated through receptors, although not the traditional 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 stereoconformation 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-$(OH)_2D_3$ (Baran et al., 1990; Okamura et al., 1974). This specificity of the nongenomic response has also been demonstrated with steroid hormones in addition to the secosteroids. 17β-, but not 17α-, estradiol elicits rapid

changes in membrane enzyme activity and fatty acid turnover in the costochondral chondrocyte cultures and alters the fluidity of isolated membranes (Schwartz et al, 1996).

Not surprisingly, more substantive changes in the chemical structure, such as the alterations in the A-ring and side chain used in the present study, elicited marked differences in biological function as well. Whereas hybrid analogue 3b regulated proteoglycan production by both types of chondrocytes, analogue 2b had no effect on proteoglycan production in either type of cell culture. These data suggest that the nature of the side chain is important in regulating biological responses. The data also indicates that analogues with even considerably modified side chains can have potent biological responses. Other hybrid analogues can be envisioned that will provide insight into structure-activity relationships.

The results of the present study confirmed previous observations demonstrating that chondrocyte response to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is cell maturation-specific. Response to the analogues also exhibited dependence on the stage of cell maturation. For example, hybrid analogue 3a had no effect on proteoglycan production in growth zone chondrocyte cultures but stimulated [^{35}S]-sulfate incorporation by resting zone cell cultures. In contrast, this hybrid analogue stimulated PKC activity in growth zone cells but had no effect on enzyme activity in resting zone cells.

These latter results may indicate that the effect of analogue 3a on proteoglycan synthesis is not mediated by PKC but through another signal transduction pathway. Although the method used measures sulfation of glycosaminoglycans, and as a result can indicate the extent of post-translational modifications of the core protein and its amino sugar side chains rather than new protein synthesis, the fact that [^{35}S]-sulfate incorporation was decreased by both cycloheximide and actinomycin D suggests that at least some of the regulation by hybrid analogues 3a and 3b

was through genomic mechanisms. Whether the protein(s) produced in response to the analogues was proteoglycan core protein or another protein(s) involved in the production of proteoglycan aggregate is not known.

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 (Ornoy et al., 1978; Lidor et al., 1987; Nakamura et al., 1992; Yamaura et al., 1993; Schwartz et al., 1995) as well as in fracture healing (Lidor et al., 1987). This study shows for the first time that some of the analogues had similar effects on chondrocyte proliferation, matrix production, and PKC activity as 24,25-$(OH)_2D_3$ whereas 1,25-$(OH)_2D_3$ did not, 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 that some of these effects are rapid, membrane-mediated nongenomic effects. The results also indicate that the analogues have potential as pharmaceuticals for treatment of specific vitamin D-related diseases which do not involve the VDR.

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VITA

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