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Membrane Actions of Vitamin D Metabolites $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ Are Retained in Growth Plate Cartilage Cells From Vitamin D Receptor Knockout Mice

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Abstract $1\alpha,25(OH)_2D_3$ regulates rat growth plate chondrocytes via nuclear vitamin D receptor (1,25-nVDR) and membrane VDR (1,25-mVDR) mechanisms. To assess the relationship between the receptors, we examined the membrane response to $1\alpha,25(OH)_2D_3$ in costochondral cartilage cells from wild type VDR(+/+) and VDR(−/−) mice, the latter lacking the 1,25-nVDR and exhibiting type II rickets and alopecia. Methods were developed for isolation and culture of cells from the resting zone (RC) and growth zone (GC, prehypertrophic and upper hypertrophic zones) of the costochondral cartilages from wild type and homozygous knockout mice. $1\alpha,25(OH)_2D_3$ had no effect on [^{3}H]-thymidine incorporation in VDR(−/−) GC cells, but it increased [^{3}H]-thymidine incorporation in VDR(+/+) cells. Proteoglycan production was increased in cultures of both VDR(−/−) and VDR(+/+) cells, based on [^{35}S]-sulfate incorporation. These effects were partially blocked by chelerythrine, which is a specific inhibitor of protein kinase C (PKC), indicating that PKC-signaling was involved. $1\alpha,25(OH)_2D_3$ caused a 10-fold increase in PKC specific activity in VDR(−/−), and VDR(+/+) GC cells as early as 1 min, supporting this hypothesis. In contrast, $1\alpha,25(OH)_2D_3$ had no effect on PKC activity in RC cells isolated from VDR(−/−) or VDR(+/+) mice and neither $1\beta,25(OH)_2D_3$ nor $24R,25(OH)_2D_3$ affected PKC in GC cells from these mice. Phospholipase C (PLC) activity was also increased within 1 min in GC chondrocyte cultures treated with $1\alpha,25(OH)_2D_3$. As noted previously for rat growth plate chondrocytes, $1\alpha,25(OH)_2D_3$ mediated its increases in PKC and PLC activities in the VDR(−/−) GC cells through activation of phospholipase A_2 (PLA_2). These responses to $1\alpha,25(OH)_2D_3$ were blocked by antibodies to 1,25-MARRS, which is a [^{3}H]-$1,25(OH)_2D_3$ binding protein identified in chick enterocytes. $24R,25(OH)_2D_3$ regulated PKC in VDR(−/−) and VDR(+/+) RC cells. Wild type RC cells responded to $24R,25(OH)_2D_3$ with an increase in PKC, whereas treatment of RC cells from mice lacking a functional 1,25-nVDR caused a time-dependent decrease in PKC between 6 and 9 min. $24R,25(OH)_2D_3$ dependent PKC was mediated by phospholipase D, but not by PLC, as noted previously for rat RC cells treated with $24R,25(OH)_2D_3$. These results provide definitive evidence that there are two distinct receptors to $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$-dependent regulation of DNA synthesis in GC cells requires the 1,25-nVDR, although other physiological responses to the vitamin D metabolite, such as proteoglycan sulfation, involve regulation via the 1,25-mVDR. J. Cell. Biochem. 90: 1207–1223, 2003.

Key words: rapid actions of steroid hormones; $1\alpha,25(OH)_2D_3$; $24R,25(OH)_2D_3$; vitamin D metabolites; VDR knockout mice; membrane receptors; growth plate chondrocytes

$1\alpha,25(OH)_2D_3$ modulates proliferation and differentiation of growth plate chondrocytes through mechanisms involving the classical nuclear vitamin D receptor (1,25-nVDR). In addition, studies examining the response of rat costochondral growth plate chondrocytes to $1\alpha,25(OH)_2D_3$ show that the effects of the vitamin D metabolite are mediated by rapid membrane-associated mechanisms. These involve signal transduction via phospholipase C (PLC) [Schwartz et al., 2003] and phospholipase

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A_2 (PLA_2) dependent pathways [Sylvia et al., 1998], resulting in protein kinase C (PKC) activation and phosphorylation of the ERK family of MAP kinases [Schwartz et al., 2002]. The membrane-dependent effects of $1\alpha,25(OH)_2D_3$ are stereospecific; $1\beta,25(OH)_2D_3$ has no effect. They can be elicited by analogues of $1,25(OH)_2D_3$ that exhibit less than 0.1% binding to the 1,25-nVDR [Boyan et al., 1997b; Greising et al., 1997; Pedrozo et al., 1999]. In addition, they can be blocked by antibodies generated to a $1\alpha,25(OH)_2D_3$-binding protein isolated from basal lateral membranes of chick intestinal epithelium [Nemere et al., 1998], termed 1,25-MARRS [Mesbah et al., 2002]. In contrast, antibodies to the 1,25-nVDR do not block these effects [Pedrozo et al., 1999]. Moreover, Western blots of extracellular matrix vesicles produced by the cells indicate that the 1,25-nVDR is not present, although these extracellular organelles contain immunoreactive protein using the anti-1,25-MARRS antibody and exhibit stereospecific responses to $1\alpha,25(OH)_2D_3$ [Pedrozo et al., 1999]. These observations indicate that the rapid responses of growth plate chondrocytes to $1\alpha,25(OH)_2D_3$ and their physiological consequences are mediated by a membrane-associated receptor (1,25-mVDR) that is different from the classical 1,25-nVDR.

Many of the physiological effects of $1\alpha,25(OH)_2D_3$ in the rat costochondral cartilage growth plate as well as in the epiphyseal growth plate are found in cells from the prehypertrophic and upper hypertrophic zones, both in vivo [Amizuka et al., 1999; Pedrozo et al., 1999; Lin et al., 2002] and in vitro [Boyan et al., 1988a]. Agents that inhibit the PLC, PKC, and PLA_2 signaling pathways, also inhibit physiological responses to the vitamin D metabolite, including [^{3}H]-thymidine incorporation, alkaline phosphatase specific activity, and [^{35}S]-sulfate incorporation [Sylvia et al., 1993, 1998; Schwartz et al., 2003]. In contrast, cells from the RC do not exhibit these responses to $1\alpha,25(OH)_2D_3$. Instead, PKC specific activity is stimulated by $24R,25(OH)_2D_3$, but PLC activity is unaffected and PLA_2 activity is decreased [Helm et al., 1996; Boyan et al., 1997a; Sylvia et al., 2001]. These effects of $24R,25(OH)_2D_3$ on signaling are stereospecific as are physiological responses to $24R,25(OH)_2D_3$ and they are inhibited by agents that block PKC but increased by agents that block PLA_2 [Helm et al., 1996; Boyan et al., 1997c]. Antibodies to 1,25-MARRS have no effect on the response of these cells to $24R,25(OH)_2D_3$. Taken together these observations suggest that $24R,25(OH)_2D_3$ exerts its effects via membrane-associated mechanisms, but by a receptor that is different from either the 1,25-mVDR or the 1,25-nVDR.

Studies in other systems support these hypotheses. Rapid membrane-dependent responses to $1\alpha,25(OH)_2D_3$ have been reported in chick intestinal epithelium [Cancela et al., 1988; Nemere and Norman, 1988; de Boland and Norman, 1998], fish enterocytes [Nemere et al., 2000], lymphocytes [Norman, 2000], and osteoblasts [Farach-Carson and Ridall, 1998; Baran et al., 2000; Boyan et al., 2002]. There is evidence that the 1,25-nVDR may associate with plasma membranes [Kim et al., 1996] and in doing so it could contribute to the rapid response. Support for this is the rapid release of Ca^{++} ions from the endoplasmic reticulum due to the interaction of occupied 1,25-nVDRs with calreticulin [Kim et al., 1996]. More recently, Baran et al. [2000a] showed that although ROS 24/1 cells lack a functional 1,25-nVDR, they exhibit a membrane response to $1\alpha,25(OH)_2D_3$. Annexin II, which is present in the membranes of ROS 24/1 cells, can bind $1\alpha,25(OH)_2D_3$. These observations indicate that at least some of the responses of cells to $1\alpha,25(OH)_2D_3$ are not via the 1,25-nVDR, and suggest that annexin II may function as a membrane receptor. However, antibodies to annexin II do not block the $1\alpha,25(OH)_2D_3$-dependent increase in PKC activity in ROS 17/2.8 osteoblast-like cells or UMR-106 osteoblast-like cells [Boyan et al., 2002a], indicating that the 1,25-mVDR responsible for PKC activation is not annexin II.

The question of whether cells possess unique receptors for $24R,25(OH)_2D_3$ is also unresolved. While it is clear that $24R,25(OH)_2D_3$ can elicit effects in cells that differ from those elicited by $1\alpha,25(OH)_2D_3$ [Pedrozo et al., 1999; Schwartz et al., 2001b; Boyan et al., 2002b] the mechanisms involved are not understood. Although specific binding for radiolabeled $24R,25(OH)_2D_3$ has been shown in growth plate chondrocytes [Somjen et al., 1982], definitive demonstration of a nuclear receptor for this vitamin D metabolite has not been shown, leading to the hypothesis that it works in some way through the 1,25-nVDR.

In order to show in a definitive manner that the regulation of PKC activity by $1\alpha,25(OH)_2D_3$

and $24R,25(OH)_2D_3$ is not via the 1,25-nVDR and that target-cell specificity of each metabolite is not limited to rats, we used growth plate chondrocytes isolated from the costochondral cartilages of wild type mice VDR(+/+) and of mice lacking a 1,25-nVDR [VDR(−/−)]. The VDR(−/−) mice, which are a model of type II vitamin D-dependent rickets, were created by targeted ablation of the second zinc finger of the 1,25-nVDR DNA binding domain [Li et al., 1997]. They do not express the gene for the 1,25-nVDR and are characterized by hypocalcemia, hyperparathyroidism, rickets, osteomalacia, and alopecia, indicating that responsiveness to $1\alpha,25(OH)_2D_3$ is impaired.

MATERIALS AND METHODS

Reagents

$1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Cell culture media and PKC assay reagents were obtained from GIBCO-BRL (Gaithersburg, MD). The PLC inhibitor U73122 and the PLA_2 inhibitor quinacrine (Quin), the PLD inhibitor wortmannin, and the PKC inhibitor chelerythrine were obtained from Calbiochem (San Diego, CA). Polyclonal rabbit antibody to the 1,25-mVDR isolated from chick intestinal epithelium basal lateral membranes (Ab99) was the gift of Dr. Ilka Nemere (Utah State University, Logan, UT). Nonspecific rabbit IgG1 was obtained from SIGMA Chemical Company (St. Louis, MO). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent obtained from Pierce Chemical Co. (Rockford, IL). [^{35}S]-sulfate and [^{3}H]-thymidine were purchased from New England Nuclear-DuPont (Boston, MA).

Chondrocyte Cultures

A mouse colony was established in the Laboratory Animal Research Center at the University of Texas Health Science Center at San Antonio using a heterozygous [VDR(+/−)] breeding pair obtained as a gift from Dr. Marie Demay (Harvard Medical School, Massachusetts General Hospital, Boston, MA). The phenotypic characteristics of these mice have been described in detail in a series of publications [Li et al., 1997; Chen et al., 2001; Donohue and Demay, 2002]. Offspring were genotyped at 2 weeks after birth. VDR(+/−) mice were allowed to breed. Homozygous wild-type [VDR(+/+)] and 1,25-nVDR knockout [VDR(−/−)] mice were euthanized when 4–5 weeks old. In addition to genotyping, homozygotes were assessed for evidence of rickets by histologic analysis of the costochondral cartilages. VDR(−/−) mice exhibited phenotypic markers of vitamin D deficiency, including rachitic growth plates with expanded hypertrophic cell zones (Fig. 1).

The mouse costochondral growth plate chondrocyte culture system used in this study was adapted from methods previously described in detail for the culture of rat costochondral growth plate chondrocytes [Boyan et al., 1988b]. Chondrocytes were obtained from the resting zone (reserve zone) and the prehypertrophic and upper hypertrophic zones (growth zone, GC) of costochondral cartilage from 4 to 5 week old male and female VDR(+/+) and VDR(−/−) mice. To accomplish this, the costochondral cartilages were removed by sharp dissection and the resting zones (RCs) and GCs were separated under a dissecting microscope. The RC was defined as the hyaline-like cartilage of the costochondral junction down to the proliferating cell zone, which marks the transition from opaque to translucent tissue. Care was taken to limit the contamination of the RC with the proliferating cell zone, but the dissection included at least one slice of the transitional region so it is likely that proliferating cell zone cells were present. The mid portion of the transitional region was discarded. The GC consisted of prehypertrophic and upper hypertrophic zone cells. To ensure that no calcifying chondrocytes or osteoblasts were included, the lower hypertrophic zone was discarded as well. By using this method, any differences in the size of the GC due to rickets in comparison with the wild type growth plate were accounted for.

Rat growth plate chondrocytes are cultured for 7–10 days at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 50 μg/ml sodium ascorbate in an atmosphere of 5% CO_2 and 100% humidity [Boyan et al. 1988b]. However, the mouse chondrocytes failed to grow under these conditions. Based on the observation that articular chondrocytes require media containing 20% FBS [Xu et al., 2001], the mouse growth plate chondrocytes were cultured in DMEM enriched with this higher concentration of serum.

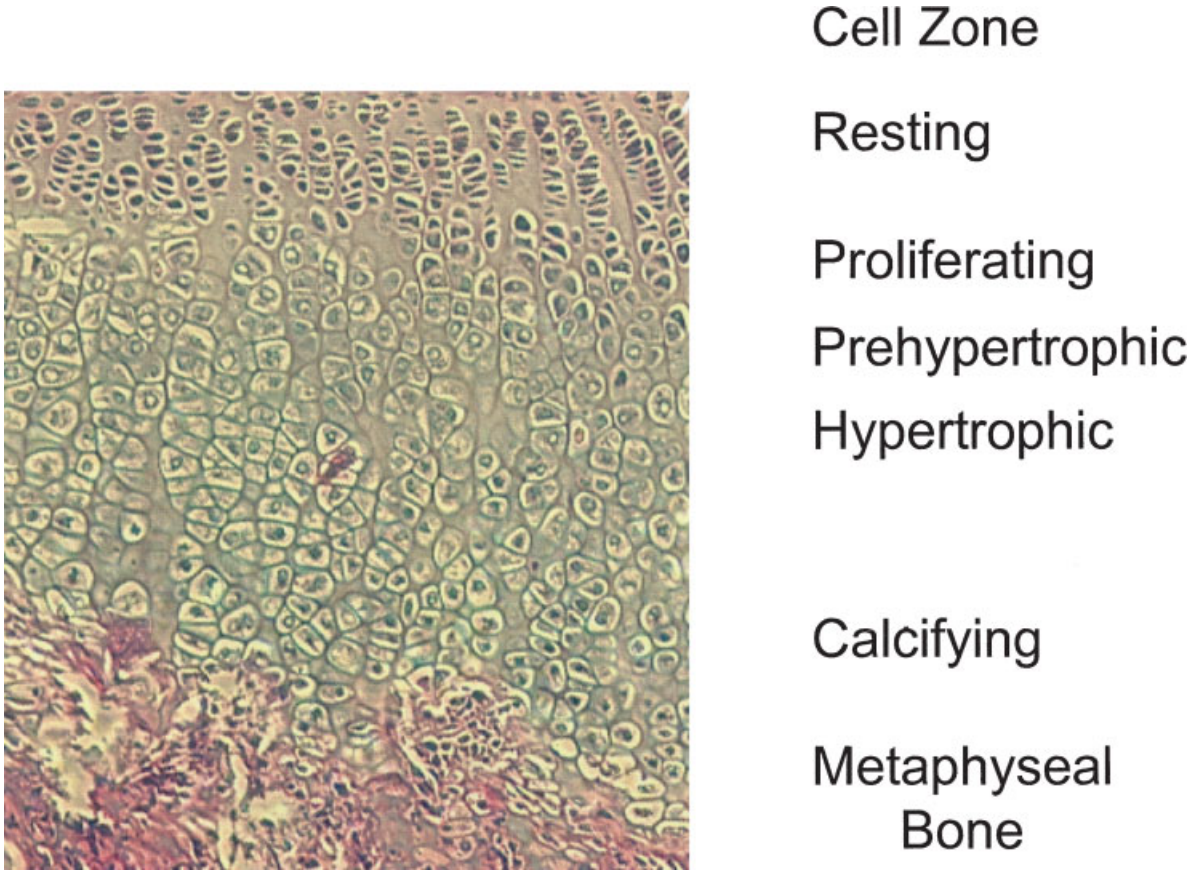

Fig. 1. Costochondral cartilage from VDR(−/−) mice demonstrating characteristics typical of rickets, including an extended hypertrophic cell zone. Sections were stained with haematoxylin and eosin. Original magnification: 10×.

Previous studies using rat chondrocytes have demonstrated a retention of differential phenotypic markers through fourth passage, including cell maturation-specific responses to $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ [Boyan et al., 1988a; Schwartz et al., 1988; Schwartz et al., 1989; Boyan et al., 1992; Boyan et al., 1997a, 2002]. It was not known if mouse chondrocytes would behave in a similar manner, but it was necessary to culture expand the cells to have enough for experimentation. Accordingly, for all experiments, confluent third passage cultures were sub-passaged into 24-well tissue culture plates and grown to confluence prior to treatment with vehicle or the appropriate experimental media. Cells were treated with vitamin D metabolites as indicated below. Metabolites were dissolved in ethanol and then diluted at least 1:5,000 in DMEM prior to being added to the cells. Any further dilutions were made using culture medium. All experiments included vehicle-only control cultures.

Regulation of PKC Activity

To determine if PKC activity was regulated by $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in the absence of a nuclear receptor for $1\alpha,25(OH)_2D_3$, GC cells and RC cells from VDR(+/+) and VDR(−/−) mice were treated with the vitamin D metabolites and PKC specific activity determined. For these experiments, confluent cultures of GC cells and RC cells were treated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ or 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$ for 9 or 90 min. To determine the time course of PKC activation, GC cells were treated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ for 1, 3, 6, 9, 12, 24, and 90 min. RC cells were treated with 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$ for the same time periods. PKC specific activity was assayed in cell layer lysates as described previously using myelin basic protein as the substrate and [^{32}P]-labeled ATP as the phosphate donor [Sylvia et al., 1993]. Assay conditions were optimized for Ca^{++}-dependent

and phospholipid-dependent PKCs such as PKCα, shown to be the PKC isoform in rat growth plate chondrocytes that is responsive to $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ [Sylvia et al., 1993].

The phospholipid signaling pathway responsible for the activation of PKC by $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in the mouse growth plate chondrocytes was examined. To determine if phosphatidylinositol-specific phospholipase C (PI-PLC) was involved, GC chondrocytes were incubated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ for 9 min. RC cells were treated with 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$ for 90 min. PLC activity was inhibited with 10 μM U73122, which is a specific inhibitor of PI-PLC [Bleasdale et al., 1989].

To verify the cell maturation specific role of PLC in the mechanism of PKC activation in VDR(−/−) GC chondrocytes, we determined if PLC was regulated by $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$. GC cells were incubated with 10^{-10}, 10^{-9}, or 10^{-8} M $1\alpha,25(OH)_2D_3$ for 9 min and RC cells were incubated with 10^{-9}, 10^{-8}, or 10^{-7} M $24R,25(OH)_2D_3$ for 90 min. Time course was assessed by determining PLC activity at 1, 3, 6, 9, 12, 24, and 90 min. PLC specific activity was measured directly using cell culture lysates as described previously [Schwartz et al., 2003]. Cell layers were harvested after the appropriate incubation time by washing with phosphate buffered saline (PBS) and loosening from the wells with a sterile cell scraper. The cell layers were then lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1% NP-40) by standing on ice for 30 min. Fifty microliter aliquots of the cell layer lysates were mixed with 50 μl of reaction buffer containing 100 mM NaCl, 0.6% sodium deoxycholate, 2 mM $CaCl_2$, 4 mM EGTA, 20 mM Tris-HCl, pH 6.0 and [^{3}H]-phosphatidylinositol 4,5-bisphosphate (PIP_2) (specific activity: 8 Ci/mmol; 50,000 cpm/reaction). The samples were incubated at 37°C for 30 min, the reaction stopped by addition of 0.1 ml of cold chloroform:methanol:12N HCl (100:100:0.6 v/v), and 50 μl of the upper aqueous layer was added to 10 ml of Ready-Gel scintillation cocktail (Beckman Coulter, Inc., Fullerton, CA) and the samples counted. Data are presented as dpm/μg cell layer protein over the 30 min time course of the incubation.

The role of phospholipase D (PLD) was assessed under the same culture conditions using 10 μM wortmannin as the inhibitor as described previously [Nakanishi et al., 1992]. Although wortmannin has been shown to inhibit phosphatidylinositol 3-phosphate at higher concentrations [Arcaro and Wymann, 1993], at the dose used in the present study, it is specific for PLD. In order to determine if PKC was modulated by a PLA_2-dependent mechanism, PLA_2 was inhibited with 10 μM Quin [Church et al., 1993] as described by us previously [Helm et al., 1996].

To test whether the effect of $1\alpha,25(OH)_2D_3$ was via the 1,25-mVDR as shown in rat growth plate chondrocytes [Schwartz et al., 2002], mouse GC chondrocytes were treated with 10^{-9} or 10^{-8} M $1\alpha,25(OH)_2D_3$ in the presence of a rabbit polyclonal antibody generated to the N-terminal amino acid sequence of the 1,25-mVDR isolated from chick enterocyte plasma membranes, termed 1,25-MARRS [Nemere et al., 2001]. While it has not been shown definitively that this protein is the 1,25-mVDR, antibodies to this synthetic peptide inhibit the rapid activation of PKC by $1\alpha,25(OH)_2D_3$ in a number of cell culture models including rat chondrocytes [Boyan et al., 1999], various osteoblast cell lines and fetal rat calvarial cells [Boyan et al., 2002a], and chick enterocytes [Nemere et al., 2001]. The antibody was used at a dilution of 1:500, as previously described [Boyan et al., 1999]. Rabbit non-specific IgG1 was used for a control.

Cell Proliferation and [^{3}H]-Thymidine Incorporation

Vitamin D_3 metabolites inhibit [^{3}H]-thymidine incorporation by rat costochondral growth plate chondrocytes [Schwartz et al., 1989]. To determine if this response to $1\alpha,25(OH)_2D_3$ and/or $24R,25(OH)_2D_3$ in mouse growth plate chondrocytes is retained in the VDR(−/−) mice, confluent cultures of GC cells were treated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ and RC cells were treated with 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$. Cell number was assessed 24 h post-confluence. 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 preconfluent cultures for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), 10^{-9}–10^{-8} M $1\alpha,25(OH)_2D_3$ for GC cell cultures or 10^{-8}–10^{-7} M $24R,25(OH)_2D_3$

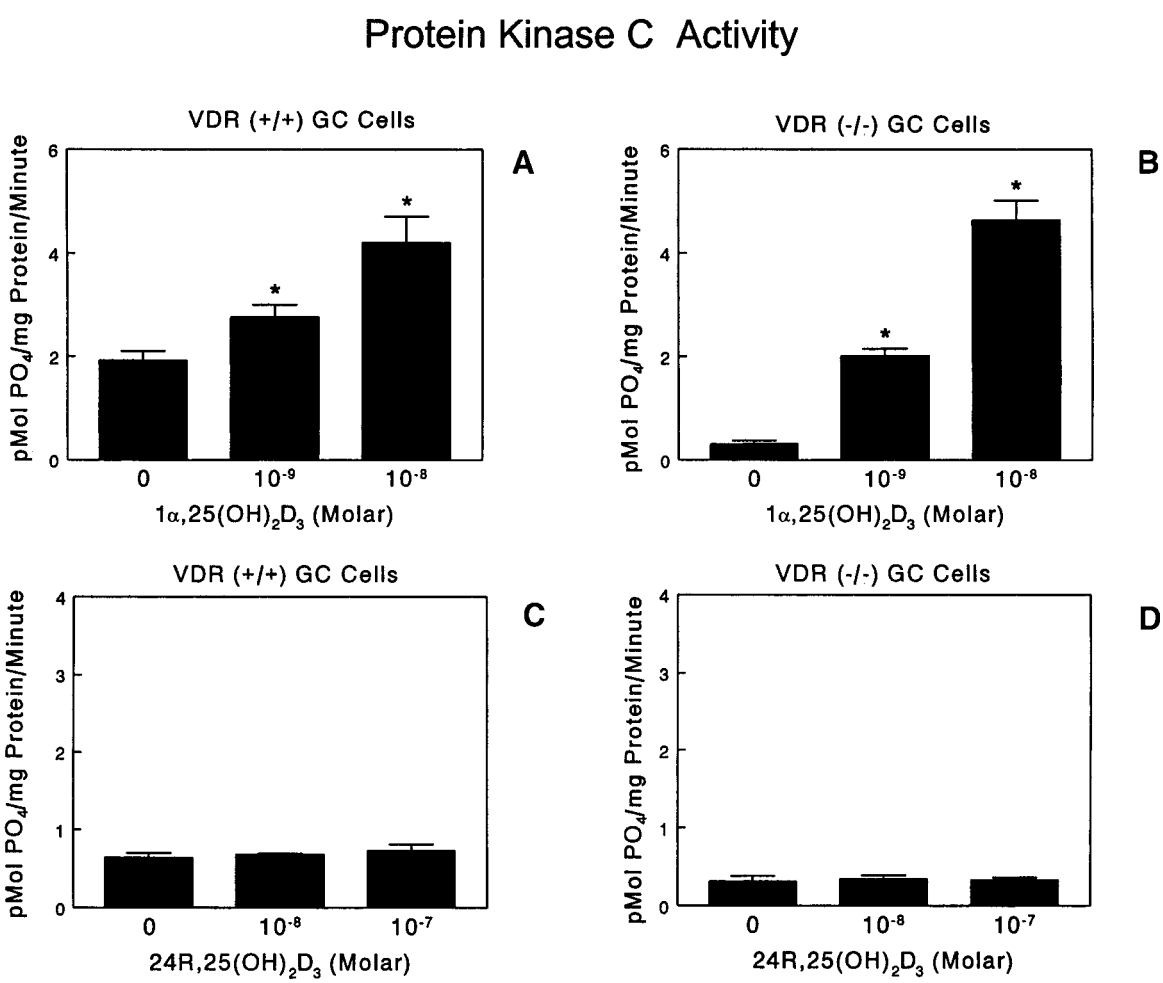

Fig. 2. Regulation of protein kinase C (PKC) specific activity in mouse VDR(+/+) and VDR(−/−) growth zone (GC) costochondral chondrocytes by $1\alpha,25(OH)_2D_3$ (**A**, **B**) and 24R, $25(OH)_2D_3$ (**C**, **D**). Confluent GC cells were treated for 9 min with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ or 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$. PKC specific activity was assayed in cell layer lysates. Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate sets of experiments, both with comparable results. *$P < 0.05$, $1\alpha,25(OH)_2D_3$ versus vehicle only.

for RC cell cultures. The cells were cultured for 24 h; 2 h prior to harvest, [^{3}H]-thymidine was added. In order to assess if any regulation of proliferation was mediated by PKC, one half of the cultures were treated with 10 μM chelerythrine, which is a general inhibitor of PKC activity [Herbert et al., 1990].

Proteoglycan Sulfation

$1\alpha,25(OH)_2D_3$ causes an increase in [^{35}S]-sulfate incorporation in rat GC chondrocyte cultures, whereas $24R,25(OH)_2D_3$ regulates proteoglycan sulfation in cultures of RC cells [Schwartz et al., 1995]. To determine if this is also the case for the mouse growth plate chondrocytes even in the absence of the 1,25-nVDR, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation by confluent cultures as described previously [O'Keefe et al., 1988; Nasatzky et al., 1993]. At confluence, fresh medium containing vehicle alone, 10^{-10}–10^{-8} M $1\alpha,25(OH)_2D_3$ for GC cell cultures or 10^{-9}–10^{-7} M $24R,25(OH)_2D_3$ for RC cell cultures, in the presence or absence of 10 μM chelerythrine, was added to the cells for 24 h. Four hours prior to harvest, 50 μl DMEM containing 18 μCi/ml [^{35}S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [^{35}S]-sulfate incorporated determined as a function of cell layer protein [Schwartz et al., 1995].

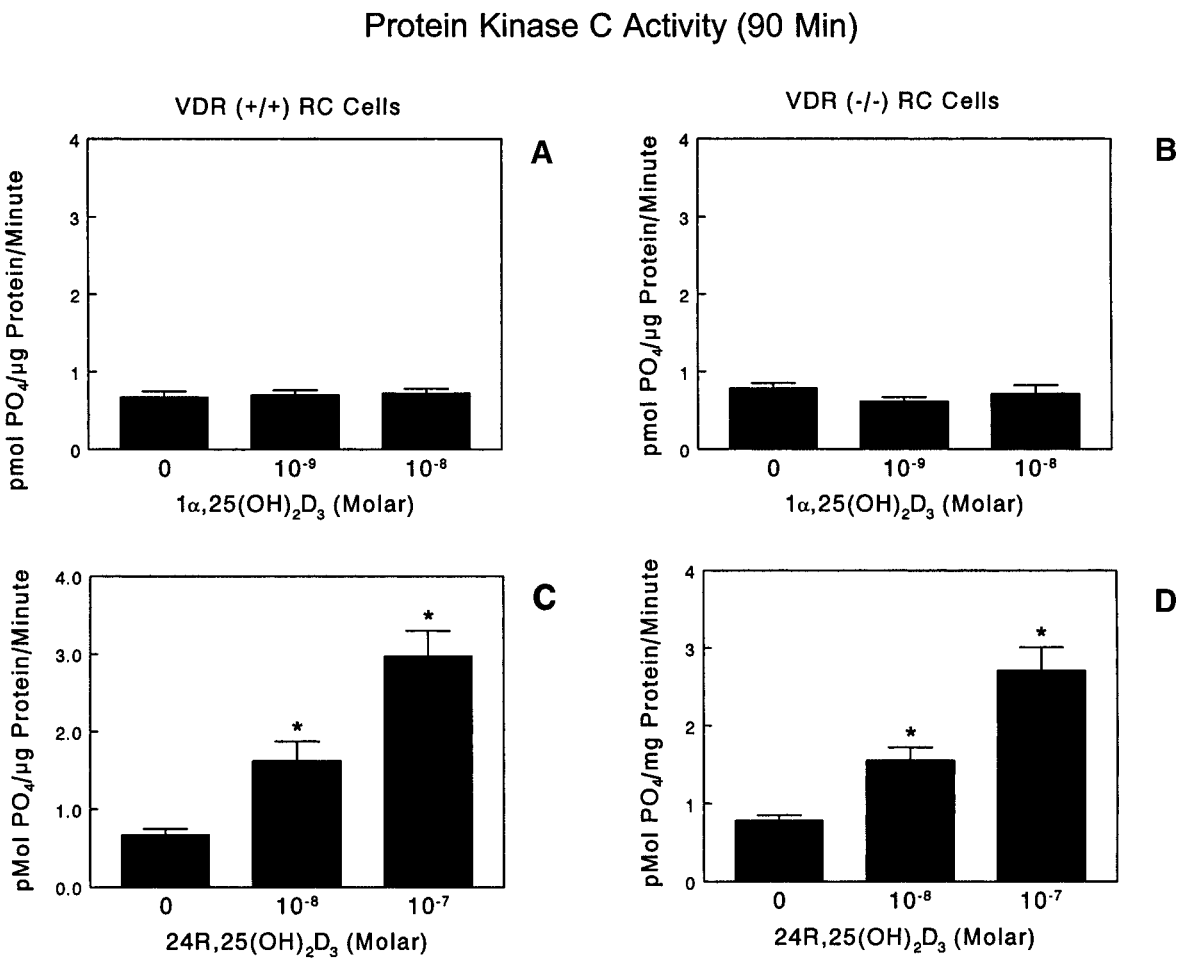

Fig. 3. Regulation of PKC specific activity in mouse VDR(+/+) and VDR(−/−) resting zone (RC) costochondral chondrocytes by 1α,25(OH)$_2$D$_3$ (**A**, **B**) or 24R,25(OH)$_2$D$_3$ (**C**, **D**). Confluent GC cells were treated for 90 min with 10^{-9} and 10^{-8} M 1α,25(OH)$_2$D$_3$ or 10^{-8} and 10^{-7} M 24R,25(OH)$_2$D$_3$. PKC specific activity was assayed in cell layer lysates. Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate sets of experiments, each with comparable results. *$P < 0.05$, 24R,25(OH)$_2$D$_3$ versus vehicle only.

Statistical Management of Data

For each experiment, each value represents the mean ± SEM of the cell layers of six individual independent cultures. Significance between groups was determined by ANOVA. Post-hoc testing was performed by using Bonferroni's modification of Student's *t*-test. *P*-values less than 0.05 were considered significant. Each experiment was repeated two or more times to ensure validity of the data. The data presented are from a single representative experiment.

RESULTS

1α,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$ regulated PKC specific activity in a cell maturation specific manner in the growth plate chondrocytes. 1α,25(OH)$_2$D$_3$ caused a dose-dependent increase in PKC specific activity in GC cells from both wild type and 1,25-nVDR knockout mice (Fig. 2A,B). Enzyme activity in control cultures of wild type cells was greater than in knockout cells at 9 min after addition of the experimental media. 10^{-9} M 1α,25(OH)$_2$D$_3$ increased PKC in wild type cells by approximately 40%. In the knockout cells, however, 10^{-9} M 1α,25(OH)$_2$D$_3$ caused a 540% increase, resulting in levels comparable to those of untreated wild type control cultures. At 10^{-8} M 1α,25(OH)$_2$D$_3$, PKC activity was maximally increased in both cell types to comparable levels, but the relative magnitude of the effect was greater in the knockout cells. 24R,25(OH)$_2$D$_3$ had no effect on PKC activity in the GC cells.

Control levels of PKC specific activity were comparable in VDR(+/+) and VDR(−/−) RC cells. No change in enzyme activity was noted in RC chondrocytes in response to $1\alpha,25(OH)_2D_3$ either at 9 min (Table I) or at 90 min (Fig. 3A,B). At 90 min after addition of experimental media, $24R,25(OH)_2D_3$ caused a dose dependent increase in PKC, which was comparable in both wild type and 1,25-nVDR knockout cells (Fig. 3C,D).

The stimulatory effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ on PKC were time-dependent as well. In wild type and knockout GC cells, $1\alpha,25(OH)_2D_3$ caused dose-dependent increases in response to $1\alpha,25(OH)_2D_3$ as early as 1 min (Fig. 4A,B). The greatest effect was seen at 6 and 9 min. By 24 min, $1\alpha,25(OH)_2D_3$ had no effect on PKC in wild type GC cells but there was a small stimulatory effect still evident in the RC cells. The increase in PKC noted in RC cells in response to $24R,25(OH)_2D_3$ was evident at 24 min in the wild type cells treated with the highest dose of the vitamin D metabolite and at 90 min at 10^{-9} and 10^{-8} M.

In RC cells from the knockout mice, the effects of $24R,25(OH)_2D_3$ on PKC were more complex (Fig. 4C,D). At 6 and 9 min, $24R,25(OH)_2D_3$ caused a small decrease in PKC. This inhibitory effect of $24R,25(OH)_2D_3$ was confirmed in a second study (Table I). However, by 24 min, PKC activity was elevated in cultures treated with 10^{-7} M. At 90 min, PKC was stimulated by 10^{-8} M $24R,25(OH)_2D_3$ and to a greater extent by 10^{-7} M $24R,25(OH)_2D_3$.

$1\alpha,25(OH)_2D_3$ regulated PKC specific activity in mouse costochondral GC cells by a mechanism involving PLC but not PLD (Fig. 5A). When VDR knockout cells were treated with 10 mM U73122 to inhibit phosphatidylinositol-specific PLC, PKC activity was reduced by 90% in control cultures, as well as in cultures treated with 10^{-9} or 10^{-8} M $1\alpha,25(OH)_2D_3$. In contrast, treatment with 10 mM wortmannin to inhibit PLD had no effect on PKC activity in control or $1\alpha,25(OH)_2D_3$-stimulated GC cell cultures.

TABLE I. The Effect of Vitamin D Metabolites PKC Activity in Resting Zone Chondrocytes From VDR Knockout and Wild Type Mice

Treatment	Knockout mice	Wild type mice
Control	0.74 ± 0.06	0.70 ± 0.08
$1\alpha,25(OH)_2D_3$ 10^{-9} M	0.67 ± 0.04	0.77 ± 0.04
$1\alpha,25(OH)_2D_3$ 10^{-8} M	0.63 ± 0.08	0.68 ± 0.08
Control	0.74 ± 0.06	0.70 ± 0.08
$24R,25(OH)_2D_3$ 10^{-8} M	0.41 ± 0.07*	0.72 ± 0.06
$24R,25(OH)_2D_3$ 10^{-7} M	0.28 ± 0.06*	0.69 ± 0.05

Confluent cultures were treated for 9 minutes with media containing vehicle or vitamin D metabolite. PKC specific activity (μmol Pi/mg protein/minute) was measured in cell layer lysates. Data are means ± SEM for 6 independent cultures and are from one of two separate experiments, both with comparable results. *$P < 0.05$, treatment v. control.

PKC specific activity in mouse RC cells was regulated by $24R,25(OH)_2D_3$ via a PLD-dependent mechanism (Fig. 5B). Inhibition of PLD with 10 mM wortmannin reduced the effect of $24R,25(OH)_2D_3$ to levels observed in the control cultures. However, inhibition of PLC with 10 mM U73122 had no effect on PKC in control cultures of VDR knockout RC cells or in cultures treated with $24R,25(OH)_2D_3$.

The differential role of PLC in mediating the effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ on GC and RC cells was confirmed in studies examining the regulation of PLC by the vitamin D metabolites (Fig. 6). $1\alpha,25(OH)_2D_3$ caused a dose-dependent increase in PLC specific activity in the 1,25-nVDR knockout GC cells (Fig. 6A). At 10^{-9} M $1\alpha,25(OH)_2D_3$, PLC activity was increased 100% and at 10^{-8} M, the increase approximated 200% over control levels. This dose-dependent stimulatory effect was evident within 1 min and was greatest at 9 min (Fig. 6B). By 24 min, only 10^{-8} M $1\alpha,25(OH)_2D_3$ was stimulatory and at 90 min, enzyme activity was at control levels. In 1,25-nVDR knockout RC cells, $24R,25(OH)_2D_3$ had no effect on PLC at any concentration (Fig. 6C) and at any time point examined (Fig. 6D).

PKC activity in the 1,25-nVDR knockout cells was regulated by $1\alpha,25(OH)_2D_3$ in GC chondrocytes and by $24R,25(OH)_2D_3$ in RC chondrocytes via mechanisms involving differential regulation of PLA_2. Inhibition of PLA_2 with 10 μM Quin had no effect on PKC activity in control cultures of GC cells but it reduced the effect of 10^{-9} M $1\alpha,25(OH)_2D_3$ to control levels and decreased the effect of 10^{-8} M $1\alpha,25(OH)_2D_3$ by more than 90% (Fig. 7A). Inhibition of PLA_2 with Quin increased PKC activity in control cultures of knockout RC cells (Fig. 7B). In cultures treated with $24R,25(OH)_2D_3$, the stimulatory effect of Quin was synergistic with the stimulatory effect of the vitamin D metabolite.

The effect of $1\alpha,25(OH)_2D_3$ on PKC in the 1,25-nVDR knockout GC cells was mediated by

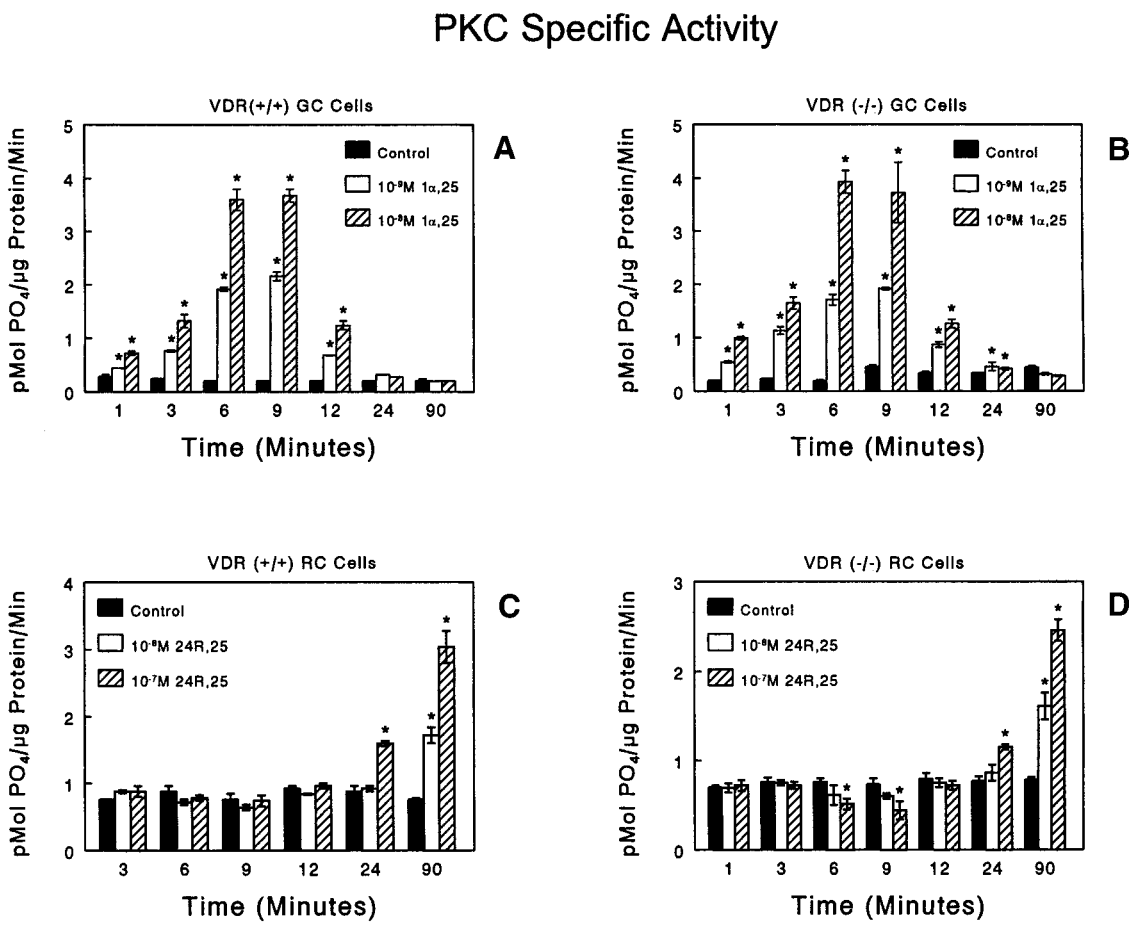

Fig. 4. Time-dependent effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ on PKC specific activity in GC (**A**, **B**) and RC (**C**, **D**) costochondral chondrocytes from VDR(+/+) and VDR(−/−) mice. Confluent GC cells were treated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$. Confluent cultures of RC cells were treated with 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$. At 1, 3, 6, 9, 12, 24 and 90 min, the cells were lysed and PKC specific activity determined in the lysates. Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate experiments, both with comparable results. $^*P < 0.05$, treatment versus control at each time point.

Ab99-immunoreactive protein. The anti-1,25-MARRS antibody had no effect on PKC activity in control cultures, but it significantly reduced the $1\alpha,25(OH)_2D_3$-dependent increase (Fig. 8). In contrast, nonspecific IgG had no effect on PKC in control cultures or in cultures treated with $1\alpha,25(OH)_2D_3$.

The 1,25-nVDR is required for regulation of proliferation by $1\alpha,25(OH)_2D_3$. Neither cell number nor [^{3}H]-thymidine incorporation was affected by the vitamin D metabolite in VDR(−/−) GC cells (Fig. 9A,B). In contrast, proliferation of VDR(+/+) GC cells was stimulated by $1\alpha,25(OH)_2D_3$ in a dose-dependent manner. This effect was mediated in part via PKC. Chelerythrine reduced the stimulatory effect of $1\alpha,25(OH)_2D_3$ on cell number and [^{3}H]-thymidine incorporation by more than 80% (Fig. 9C,D).

Synthesis of sulfated glycosaminoglycans in response to $1\alpha,25(OH)_2D_3$ did not require the 1,25-nVDR. $1\alpha,25(OH)_2D_3$ caused a dose-dependent increase in [^{35}S]-sulfate incorporation by cultures of VDR(−/−) GC cells (Fig. 10A). A similar increase was noted in wild type GC chondrocyte cultures (Fig. 10B). The stimulatory effect of $1\alpha,25(OH)_2D_3$ was mediated by PKC. Chelerythrine reduced the stimulatory effect of $1\alpha,25(OH)_2D_3$ in both wild type and 1,25-nVDR knockout cells by more than 90%.

DISCUSSION

These studies provide the first definitive evidence that $1\alpha,25(OH)_2D_3$ can regulate growth plate chondrocytes in a cell maturation specific manner through mechanisms that do not

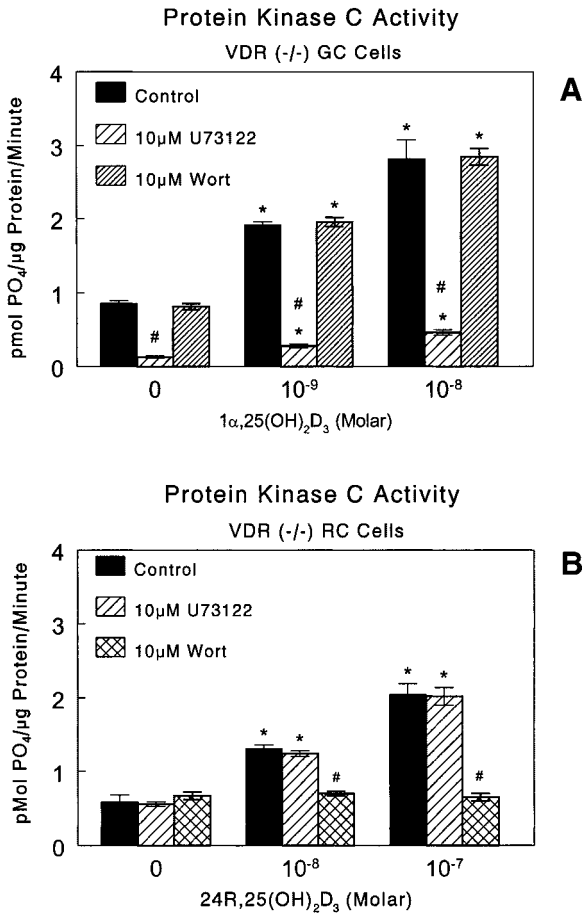

Fig. 5. Effect of phospholipase C (PLC) and phospholipase D (PLD) inhibition on PKC specific activity in mouse VDR(−/−) GC (**A**) and RC (**B**) costochondral chondrocytes. GC cells were treated for 9 min with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$. PLC was inhibited with 10 μM U73122 and PLD was inhibited with 10 μM wortmannin (Wort). Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate experiments, both with comparable results. *$P < 0.05$, treatment versus vehicle control in the absence of any vitamin D metabolite; #$P < 0.05$, inhibitor versus control at each concentration of vitamin D metabolite.

involve the 1,25-nVDR. Moreover, they show for the first time that growth plate chondrocytes can respond to $24R,25(OH)_2D_3$ in the absence of the 1,25-nVDR and they confirm that $24R,25(OH)_2D_3$ specifically targets cells from the RC of the growth plate whereas $1\alpha,25(OH)_2D_3$ specifically targets cells from the GC. This study also describes the first successful culture of growth plate chondrocytes from mice and shows that they behave in culture like rat growth plate chondrocytes with respect to $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$. Finally, the results demonstrate that some physiological responses of growth plate chondrocytes to $1\alpha,25(OH)_2D_3$, such DNA synthesis, depend on the 1,25-nVDR, but mechanisms involving PKC signaling are involved, based on reduction in [^{3}H]-thymidine incorporation by VDR(−/−) GC cells. Other physiological responses to $1\alpha,25(OH)_2D_3$ that have been ascribed to the 1,25-nVDR, such as synthesis of sulfated glycosaminoglycans, may actually be mediated wholly or in part by the 1,25-mVDR.

There is considerable evidence that the rapid response to $1\alpha,25(OH)_2D_3$ is via a membrane-associated mechanism that appears to be receptor-mediated. In rat growth plate chondrocytes, activation of PKCα is limited to cells from the GC [Sylvia et al., 1993]. It is metabolite-specific and stereospecific [Sylvia et al., 1994]; it involves a complex signaling cascade initiated by activation of PLA_2 and PI-PLC, but not by PLD [Schwartz et al., 2003]; and it can be blocked by antibodies generated to 1,25-MARRS [Nemere et al., 1998]. The present study indicates that this same mechanism modulates the rapid effects of $1\alpha,25(OH)_2D_3$ on mouse growth plate chondrocytes. PKC specific activity was increased by $1\alpha,25(OH)_2D_3$ only in cells from the GC; $1\beta,25(OH)_2D_3$ had no effect on PKC demonstrating stereospecificity; moreover, $24R,25(OH)_2D_3$ had no effect on PKC in these cells. Inhibition of PLA_2 and PLC blocked PKC stimulation by $1\alpha,25(OH)_2D_3$ whereas inhibition of PLD had no effect. In addition, PKC stimulation by $1\alpha,25(OH)_2D_3$ could be blocked with antibodies to the chick 1,25-MARRS.

If anything, the mouse response to $1\alpha,25(OH)_2D_3$ is more robust. In rat GC chondrocytes, the greatest effect of $1\alpha,25(OH)_2D_3$ on PKC is seen at 9 min although increases are evident within 3 min. Dose dependent increases in PKC specific activity in the mouse chondrocytes occurred within 1 min and peak increases occurred by 6 min. Moreover, this mechanism operates in the absence of the 1,25-nVDR, since all components of the signaling cascade were functional in cells from the VDR(−/−) mice.

The observation that $1\alpha,25(OH)_2D_3$ can elicit a rapid response in the absence of a functional 1,25-nVDR is not unique to this study. Transfection of ROS 24/1 rat osteoblast-like cells, which lack a functional 1,25-nVDR, with annexin II restores transcaltachia in response to $1\alpha,25(OH)_2D_3$ [Baran et al., 2000b]. Moreover, we have shown that when extracellular matrix vesicles are incubated directly

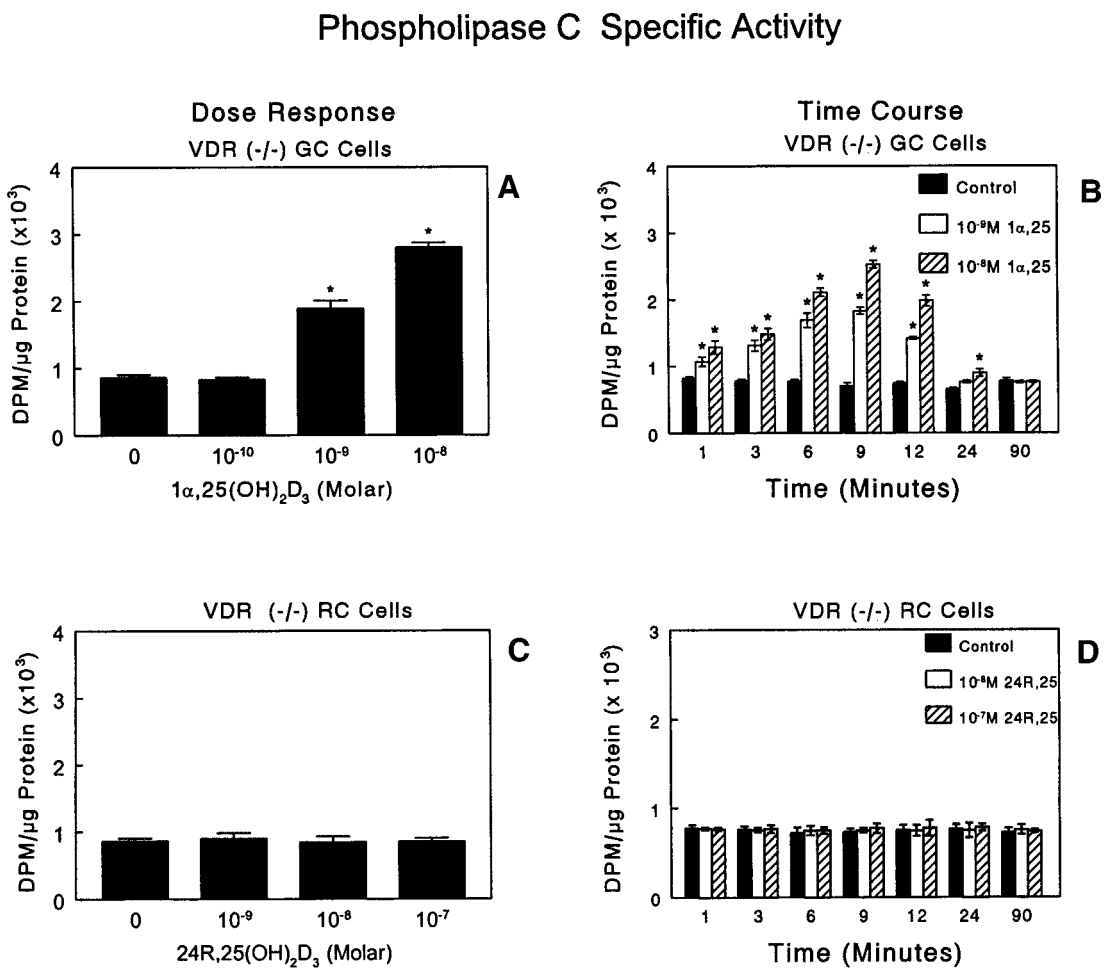

Fig. 6. Regulation of PLC specific activity in VDR(−/−) mouse GC and RC costochondral chondrocytes by 1α,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$. GC cells were treated for 9 min with 10^{-10} to 10^{-8} M 1α,25(OH)$_2$D$_3$ (**Panel A**). Time course was assessed in GC cultures treated for 1, 3, 6, 9, 12, 24 and 90 min with 10^{-9} and 10^{-8} M 1α,25(OH)$_2$D$_3$ (**Panel B**). RC cells were treated for 90 min with 10^{-9} and 10^{-7} M 24R,25(OH)$_2$D$_3$ (**Panel C**). Time course was assessed at 1, 3, 6, 9, 12, 24 and 90 min in cultures treated with 10^{-8} and 10^{-7} M 24R,25(OH)$_2$D$_3$ (**Panel D**). Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate experiments, each with comparable results. *$P < 0.05$, treatment versus vehicle control at each time point.

with 1α,25(OH)$_2$D$_3$, PKC activity is regulated although no 1,25-nVDR is present and there is no possibility of gene transcription or protein synthesis [Schwartz et al., 2001a]. This is the first demonstration, however, that PKC is regulated by 1α,25(OH)$_2$D$_3$ in intact chondrocytes that lack a functional 1,25-nVDR.

As noted previously for rat growth plate chondrocytes [Sylvia et al., 1993], response to 24R,25(OH)$_2$D$_3$ in the mouse costochondral cartilage is targeted to cells from the RC. 1α,25(OH)$_2$D$_3$ had no effect on PKC in these cells but 24R,25(OH)$_2$D$_3$ elicited a dose-dependent increase at the same time reported for the rat cells. The absence of a response to 1α,25(OH)$_2$D$_3$ precludes the hypothesis that 1α,25(OH)$_2$D$_3$ initiates a cascade leading to up-regulation of a 24R,25(OH)$_2$D$_3$-sensitive receptor. Moreover, the retention of the stimulatory effect of 24R,25(OH)$_2$D$_3$ on PKC specific activity in the VDR(−/−) mice provides definitive evidence that at least some of the responses to this metabolite are not mediated through the 1,25-nVDR, as has also been suggested by others [de Boland and Norman, 1990; de Boland et al., 1994, 1996; Seo et al., 1996; Asada et al., 2001; Nemere et al., 2003].

VDR(−/−) RC cells exhibited an unexpected rapid decrease in PKC activity in response to 24R,25(OH)$_2$D$_3$ that was not evident in wild

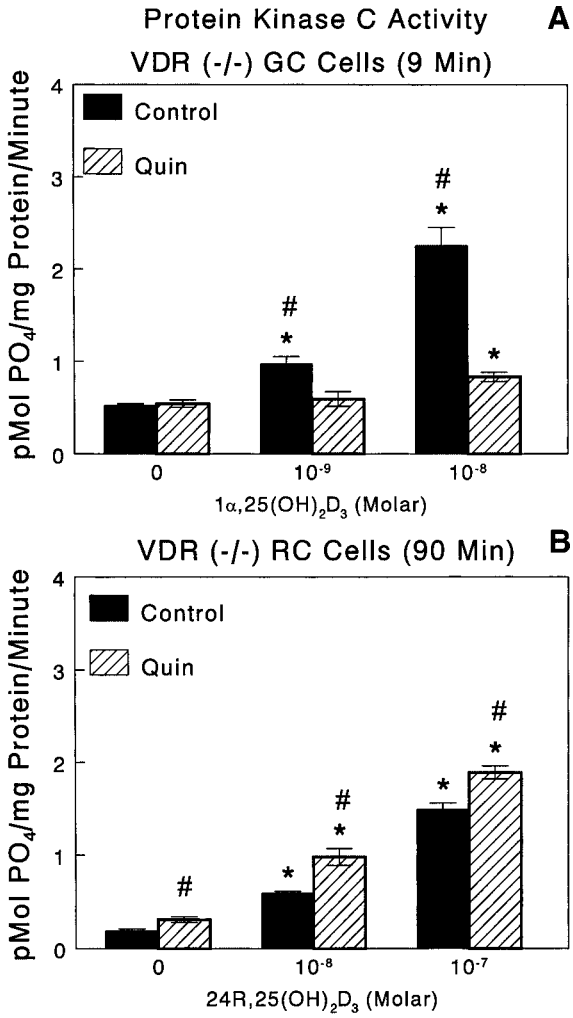

Fig. 7. Effect of phospholipase A_2 (PLA_2) inhibition on PKC specific activity in GC and RC costochondral chondrocytes from VDR(−/−) mice. GC cells were treated with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$ for 9 min (**A**). RC cells were treated with 10^{-7} and 10^{6} M $24R,25(OH)_2D_3$ for 90 min (**B**). PLA_2 was inhibited with 10 μM quinacrine (Quin). PKC was assessed in cell layer lysates. Values are mean ± SEM for N = 6 independent cultures per variable. Data are from one of two separate experiments, both with comparable results. $^*P < 0.05$, treatment versus vehicle control; $\#P < 0.05$, with Quin versus without Quin.

type cells. This suggests that the 1,25-nVDR to some extent regulates PKC in these cells. The culture media contained high levels of serum to ensure viability of the cells. However, serum contains low levels of $1\alpha,25(OH)_2D_3$ ($<10^{-12}$ M), which may help to maintain constitutive levels of PKC in RC cells through a 1,25-nVDR-dependent mechanism. The fact that the decrease in response to $24R,25(OH)_2D_3$ occurs at 6 and 9 min only suggests that the vitamin D metabolite may have down-regulated the baseline pool of enzyme that was activated in response to the fresh media added at time zero. RC cells possess membrane receptors for $1\alpha,25(OH)_2D_3$ [Nemere et al., 1998] based on specific binding of [^{3}H]-$1\alpha,25(OH)_2D_3$ and evidence of protein that cross reacts with the anti-1,25-MARRS antibody on Western blots of matrix vesicles produced by the cells, but they were presumed to be inactive. It is only in the total absence of any 1,25-nVDR that even this low level of rapid PKC sensitivity to $1\alpha,25(OH)_2D_3$ was evident. Interestingly, $24R,25(OH)_2D_3$ does cause a rapid increase in PKC activity when incubated directly with plasma membranes isolated from rat RC chondrocyte cultures [Sylvia et al., 1997], indicating that the decrease noted in the VDR(−/−) cells was not due to membrane-associated enzyme.

$24R,25(OH)_2D_3$ stimulates PKC activity in both the wild type and 1,25-nVDR knockout cells via a mechanism involving PLD, but not PLC or PLA_2, as has been noted previously for the rat RC chondrocytes [Sylvia et al., 2001]. This suggests that $24R,25(OH)_2D_3$ acts via a different receptor than $1\alpha,25(OH)_2D_3$ but it does not preclude that both metabolites use the same receptor. The physicochemical properties of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ are quite different [Seo et al., 1996; Kato et al., 1998], and they could interact with the same receptor in different ways. Arguing against this is report of a specific binding protein for $24R,25(OH)_2D_3$ isolated from chick lysosomal membranes [Nemere et al., 2003].

This study also shows that physiological responses of mouse growth plate chondrocytes to $1\alpha,25(OH)_2D_3$ are regulated by a variety of mechanisms. The proliferative effect of $1\alpha,25(OH)_2D_3$ on GC cells requires the action of the 1,25-nVDR, since VDR(−/−) cells exhibits no change in proliferation at any concentration examined. In contrast, VDR(+/+) cells responded to $1\alpha,25(OH)_2D_3$ with a dose-dependent increase in cell number and DNA synthesis. At least part of this effect in the wild-type cells is due to a mechanism that requires PKC. This suggests that the 1,25-mVDR plays a role but it is not sufficient in itself to elicit a proliferative response. An alternative explanation is the action of the 1,25-nVDR is potentiated by a PKC-dependent mechanism that is independent of the 1,25-mVDR. Studies examining proliferation of rat GC cells in the presence of blocking antibodies to the

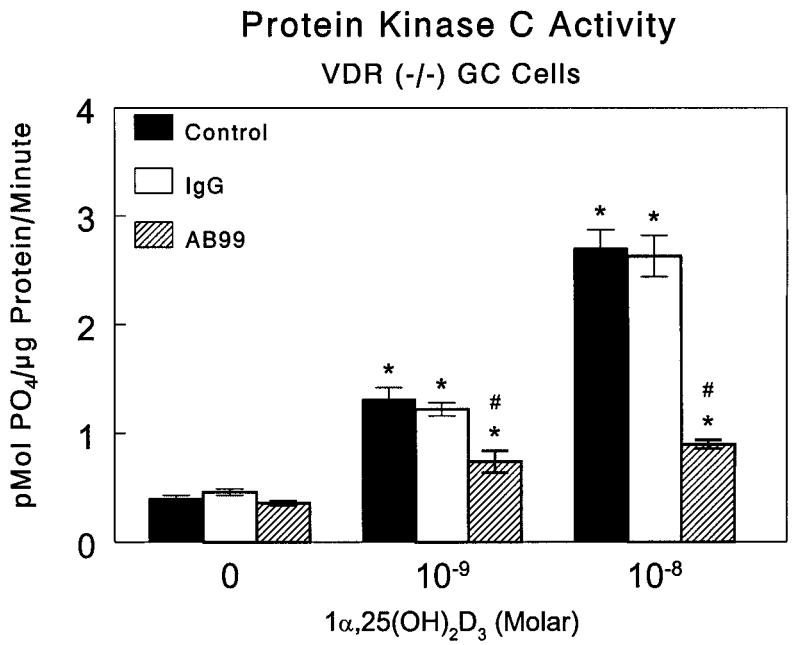

Fig. 8. 1α,25(OH)$_2$D$_3$-dependent regulation of PKC specific activity in VDR(−/−) mouse GC costochondral chondrocytes involves the 1,25-m VDR. GC cells were treated for 9 min with 10^{-9} and 10^{-8} M 1α,25(OH)$_2$D$_3$. Signaling through the 1,25–mVDR was blocked using A699 (1:500 dilution), a rabbit polyclonal antibody that was generated to the N-terminal peptide of the click enterocyte 1,25-mVDR. Cultures were also treated with a 1:500 v/v dilution of rabbit IgG. Values are mean ± SEM for N = 6 independent cultures for each variable. Data are from one of two separate experiments, both with comparable results. *$P < 0.05$, treatment versus vehicle control in the absence of 1α,25(OH)$_2$D$_3$; #$P < 0.05$, 1α,25(OH)$_2$D$_3$ versus no 1α,25(OH)$_2$D$_3$.

1,25-mVDR indicate that the proliferative response is attenuated [Pedrozo et al., 1999], implicating the membrane receptor in the mechanism.

This appears to be the case for proteoglycan synthesis as well. 1α,25(OH)$_2$D$_3$ caused a dose-dependent increase in [^{35}S]-sulfate incorporation in both VDR(+/+) and VDR(−/−) cells, indicating that the 1,25-nVDR was not required. The response to the vitamin D metabolite was reduced by antibodies to the 1,25-mVDR, but not totally blocked. It is possible that the antibody titer necessary to completely prevent receptor activation was not achieved. Other signaling pathways may also play a role.

While this study shows that rapid responses to 1α,25(OH)$_2$D$_3$ can occur in the absence of the 1,25-mVDR, they do not address the identity of this putative receptor. The anti-1,25-MARRS antibody blocks the stimulatory effect of 1α,25(OH)$_2$D$_3$ on PKC in the VDR(−/−) GC cells, as noted previously for 1α,25(OH)$_2$D$_3$-activated PKC in rat costochondral GC cells [Nemere et al., 1998] and several different osteoblast-like cell lines [Boyan et al., 2002a]. However, the characteristics of this protein have not been described and it is not yet clear if it is the only membrane associated receptor for this secosteroid, or if it is indeed a receptor in the tradition of other membrane receptors that signal via PKC. Studies using VDR null mice in which the zinc finger of the VDR was disrupted show that both genomic and nongenomic responses to 1α,25(OH)$_2$D$_3$ were abrogated [Erben et al., 2002]. This suggests that at least for the parameters measured by these investigators, the traditional 1,25-nVDR can participate in both kinds of signaling. The 1,25-nVDR has been shown to associate with plasma membranes in osteoblast-like cells [Kim et al., 1996], supporting this hypothesis. At least some of the membrane associated responses to 1α,25(OH)$_2$D$_3$ are not due to the 1,25-nVDR, however. Matrix vesicles isolated from chondrocyte cultures [Nemere et al., 1998; Schwartz et al., 2002] and osteoblast-like cell cultures [Boyan et al., 2002a] lack any evidence of a nuclear VDR, yet they exhibit direct nongenomic responses to 1α,25(OH)$_2$D$_3$. The

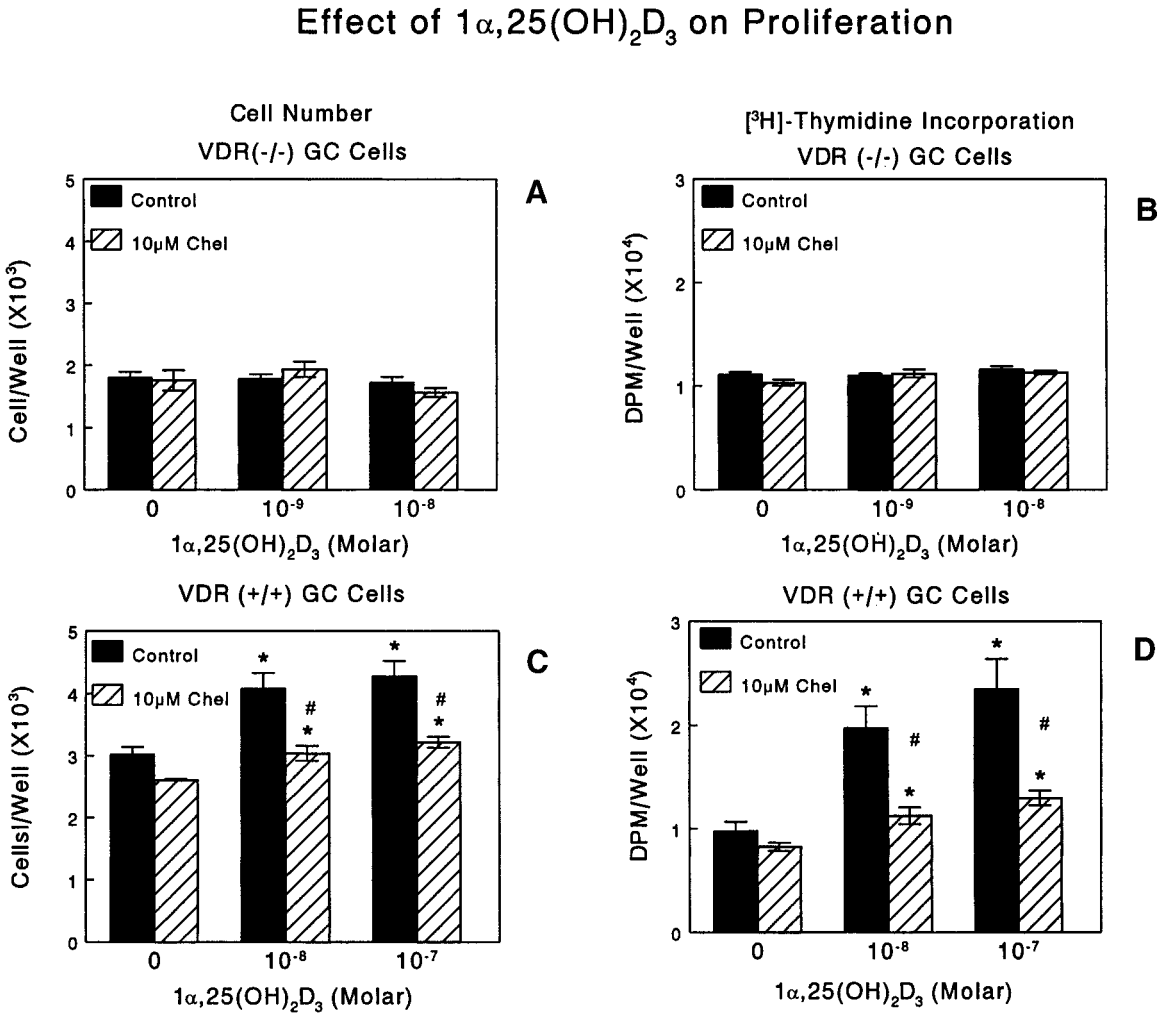

Fig. 9. 1α,25(OH)$_2$D$_3$-dependent regulation of proliferation in mouse costochondral GC chondrocytes. Confluent cultures of VDR(−/−) and VDR(+/+) GC cells were treated with 10^{-9} and 10^{-8} M 1α,25(OH)$_2$D$_3$ for 24 h. Four hours prior to harvest, [^{3}H]-thymidine was added to one half of the cultures (**Panels B, D**). Cell number was determined in the other half of the cultures (**Panels A, C**). To assess the role of PKC in 1α,25(OH)$_2$D$_3$ regulated proliferation, PKC was inhibited using 10 μM chelerythrine (Chel). Values are mean ± SEM for N = 6 independent cultures for each variable. Data are from one of two separate experiments, both with comparable results. *$P < 0.05$, 1α,25(OH)$_2$D$_3$ versus no 1α,25(OH)$_2$D$_3$; #$P < 0.05$, with chelerythrine versus no chelerythrine.

retention of a functional 1,25-mVDR in the absence of a functional 1,25-nVDR may also contribute to the healing of rachitic cartilage that occurs when normocalcemia is restored in the VDR(−/−) mice [Amling et al., 1999], since many of the matrix vesicle characteristics associated with initiation of mineral deposition in the growth plate, such as alkaline phosphatase activity, are regulated by this pathway [Pedrozo et al., 1999].

In summary, the VDR(−/−) mouse model used in this study has enabled us to clearly define the importance of the 1,25-mVDR to the response of chondrocytes in the growth plate to 1α,25(OH)$_2$D$_3$. The mechanisms involved are consistent with those found in rat growth plate chondrocytes supporting the PLA$_2$/PLC/PKC signaling pathway as fundamental to the action of the 1,25-mVDR. In addition, this model shows in a definitive manner that 24R,25(OH)$_2$D$_3$ targets resting zone cells, activating PKC through a non-1,25-nVDR mechanism and by a pathway involving PLD. Some but not all of the physiological responses of growth plate chondrocytes to 1α,25(OH)$_2$D$_3$ are mediated by the 1,25-mVDR, and the action of

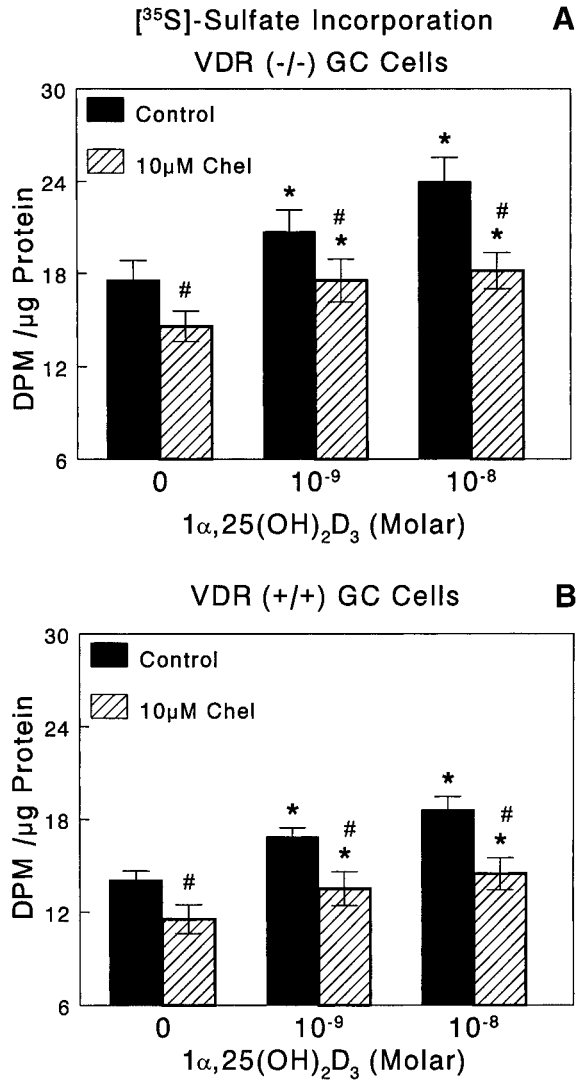

Fig. 10. $1\alpha,25(OH)_2D_3$-dependent regulation of $[^{35}S]$-sulfate incorporation in confluent cultures of VDR(−/−) (**A**) and VDR(+/+) (**B**) mouse GC costochondral chondrocytes. GC cells were treated for 24 h with 10^{-9} and 10^{-8} M $1\alpha,25(OH)_2D_3$. Prior to harvest, $[^{35}S]$-sulfate was added to the culture media. To assess whether the effect of $1\alpha,25(OH)_2D_3$ was mediated by PKC, one half of the cultures was treated with 10 μM chelerythrine (Chel). Values are mean ± SEM for N = 6 independent cultures for each variable. Data are from one of two separate experiments, both with comparable results. $^*P < 0.05$, $1\alpha,25(OH)_2D_3$ versus no $1\alpha,25(OH)_2D_3$; $\#P < 0.05$, with Chel versus no Chel.

this receptor potentiate responses mediated by the 1,25-nVDR.

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