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# Mechanisms regulating differential activation of membrane-mediated signaling by 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ ☆

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**Abstract**

Vitamin D metabolites 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ regulate endochondral ossification in a cell maturation-dependent manner via membrane-mediated mechanisms. 24*R*,25(OH)$_2$D$_3$ stimulates PKC activity in chondrocytes from the growth plate resting zone, whereas 1α,25(OH)$_2$D$_3$ stimulates PKC in growth zone chondrocytes. We used the rat costochondral growth plate cartilage cell model to study how these responses are differentially regulated. 1α,25(OH)$_2$D$_3$ acts on PKC, MAP kinase, and downstream physiological responses via phosphatidylinositol-specific PLC-β; 24*R*,25(OH)$_2$D$_3$ acts via PLD. In both cases, diacylglycerol (DAG) is increased, activating PKC. Both cell types possess membrane and nuclear receptors for 1α,25(OH)$_2$D$_3$, but the mechanims that render the 1α,25(OH)$_2$D$_3$ pathway silent in resting zone cells or the 24*R*,25(OH)$_2$D$_3$ pathway silent in growth zone cells are unclear. PLA$_2$ is pivotal in this process. 1α,25(OH)$_2$D$_3$ stimulates PLA$_2$ activity in growth zone cells and 24*R*,25(OH)$_2$D$_3$ inhibits PLA$_2$ activity in resting zone cells. Both processes result in PKC activation. To understand how negative regulation of PLA$_2$ results in increased PKC activity in resting zone cells, we used PLA$_2$ activating peptide to stimulate PLA$_2$ activity and examined cell response. PLAP is not expressed in resting zone cells in vivo, supporting the hypothesis that PLA$_2$ activation is inhibitory to 24*R*,25(OH)$_2$D$_3$ action in these cells.


*Keywords:* 1α,25(OH)$_2$D$_3$; 24*R*,25(OH)$_2$D$_3$; Phospholipase A$_2$; Phospholipase A$_2$ activating protein (PLAP); Protein kinase C (PKC); Phospholipase C (PLC); Chondrocytes

## 1. Introduction

We have used cells from the rat costochondral growth plate cartilage as a model to study the rapid actions of 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ in vivo and in vitro [1]. The growth plate is particularly useful because of its distinctive morphology. Chondrocytes align in columns, each column representing the lineage cascade of one clonal population. Cells in the resting zone are surrounded by a proteoglycan rich extracellular matrix that does not support calcification. These cells respond primarily to 24*R*,25(OH)$_2$D$_3$. 24*R*,25(OH)$_2$D$_3$ causes an increase in protein kinase C (PKC) activity within 3 min but maximal activity is not seen until 90 min. The mechanism involves a decrease in phospholipase A$_2$ (PLA$_2$) activity, reducing the levels of arachidonic acid and ultimately of prostaglandin, both of which are inhibitory to PKC activation in these cells. In addition, 24*R*,25(OH)$_2$D$_3$ causes an increase in phospholipase D (PLD), resulting in diacylglycerol (DAG) production and PKC activation, but not translocation.

In response to signals not yet well understood, resting zone cells undergo a set number of cell divisions and then enter a maturation state that is characterized by rapid hypertrophy culminating in calcification of the extracellular matrix. Cells from the prehypertrophic and upper hypertrophic zones (growth zone) respond primarily to 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ causes an increase in PKC within 1 min that is maximal at 9 min. The mechanism involves a rapid increase in PLA$_2$ activity and the products of PLA$_2$ action initiate signaling cascades that contribute to the PKC increase. Arachidonic acid activates PKC directly and serves as a substrate for cyclooxygenase-1 (Cox-1), producing prostaglandin. One of the prostaglandins, PGE$_2$, participates in the mechanism via its EP1 receptor. Phospholipase C (PLC) also plays a role in the activation of PKC by

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Table 1
Osteoblast maturation state determines responsiveness to 1α,25$(OH)_2D_3$ and/or 24*R*,25$(OH)_2D_3$

| Cell type | Osteoblast maturation state | 1α,25 $(OH)_2D_3$ | 24*R*,25 $(OH)_2D_3$ |
|---|---|---|---|
| 2T3 cells | Pre-osteoblasts | | X |
| 2T3 cells + BMP-2 | Osteoblasts | X | |
| FRC cells | Multipotent pre-osteoblasts | | X |
| FRC cells + BMP-2 | Osteoblasts | X | |
| MG63 cells | Immature osteoblasts | | |
| ROS 17/2.8 cells | Osteoblasts | X | x |
| MC-3T3-E1 cells | Mature osteoblasts | X | |
| UMR-106 cells | Mature osteoblasts | X | |
| MLO-Y4 cells | Osteocyte-like | X | |

Confluent cultures of osteoblast cell lines were treated for 9 or 90 min with $10^{-10}$ to $10^{-7}$ M 1α,25$(OH)_2D_3$ or $10^{-9}$ to $10^{-6}$ M 24*R*,25$(OH)_2D_3$. PKC specific activity was determined in cell layer lysates using an immunoassay kit. In addition, confluent cultures of pre-osteoblastic mouse 2T3 cells and fetal rat calvarial (FRC) cells were cultured for ≥7 days with rhBMP-2 before treatment with the Vitamin D metabolites as described above.

1α,25$(OH)_2D_3$, increasing DAG and inositol trisphosphate (IP3) levels. Once DAG binds to PKC, translocation to the plasma membrane occurs.

These studies suggest that differential responsiveness to 1α,25$(OH)_2D_3$ or 24*R*,25$(OH)_2D_3$ is related to cell matuation state. To verify that this is indeed the case, we examined a number of osteoblast cell lines at various states of maturation in the osteoblast linage for evidence of PKC activation by one or both of these Vitamin D metabolites [2]. Cells that exhibited an increase in PKC activity in response to 24*R*,25$(OH)_2D_3$ have been described as relatively immature osteoblasts, whereas those cell lines that responded to 1α,25$(OH)_2D_3$ have been defined as relatively mature osteoblasts (Table 1). Moreover, pre-osteoblastic 2T3 cells did not respond to 1α,25$(OH)_2D_3$ unless they were pretreated with BMP-2 for 7 days to induce osteoblastic differentiation. Similarly, confluent cultures of fetal rat calvarial cells required BMP-2 treatment for a minimum of 7 days before 1α,25$(OH)_2D_3$ caused an increase in PKC. Prior to this time, 2T3 cells and fetal rat calvarial cells exhibted increased PKC in response to 24*R*,25$(OH)_2D_3$.

## 2. Membrane receptors for 1α,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$

To better understand the mechanisms involved in the transition from 24*R*,25$(OH)_2D_3$ responsiveness to 1α,25$(OH)_2D_3$ responsiveness, we returned to an examination of the rat costochondral growth plate cartilage cell model. Both resting zone and growth zone cells possess nuclear receptors for 1α,25$(OH)_2D_3$ and both respond to 1α,25$(OH)_2D_3$ with a decrease in cell number and [$^3$H]-thymidine incorporation [3]. However, only growth zone cells exhibit rapid increases in PKC when treated with 1α,25$(OH)_2D_3$. Moreover, only resting zone cells exhibit a rapid PKC response to 24*R*,25$(OH)_2D_3$, although specific binding of radiolabeled 24*R*,25$(OH)_2D_3$ is present throughout the growth plate [4], suggesting that receptors for this metabolite are present as well. In growth zone cells treated with 1α,25$(OH)_2D_3$ and in resting zone cells treated with 24*R*,25$(OH)_2D_3$, there is an increase in mitogen activated protein (MAP) kinase activity commensurate with the change in PKC described above [5]. The metabolite-specific activation of MAP kinase explains how the physiological responses of the resting zone and growth zone chondrocytes are affected at the genomic level by the target cell-specific rapid actions of the metabolites at the membrane. What is not clear is why resting zone cells and growth zone cells respond so differently to 1α,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$. This paper reviews our studies to resolve the perplexing question of why 1α,25$(OH)_2D_3$ is silent in resting zone cells and 24*R*,25$(OH)_2D_3$ is silent in growth zone cells with respect to PKC activation and its downstream biological consequences.

Our observations support the hypothesis that receptors for 1α,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$ are present in the membranes of resting zone and growth zone cells. Binding studies using radiolabeled Vitamin D metabolites show that specific and saturable binding is present in the target cell for each metabolite. However, specific binding for 1α,25$(OH)_2D_3$ was also found in plasma membranes and matrix vesicles isolated from resting zone cells and binding for 24*R*,25$(OH)_2D_3$ was also found in plasma membranes and matrix vesicles from growth zone cells [6,7]. Polyclonal antibodies generated to the N-terminal amino acid sequence of the 1α,25$(OH)_2D_3$-binding protein isolated from chick intestinal basal lateral membranes (Ab99), blocked PKC activation by 1α,25$(OH)_2D_3$ in growth zone cells and 1α,25$(OH)_2D_3$-dependent inhibition of PKC in matrix vesicles isolated from growth zone cell cultures. However, this antibody had no effect on PKC in resting zone cells or isolated matrix vesicles, whether they were treated with 1α,25$(OH)_2D_3$ or 24*R*,25$(OH)_2D_3$. Western blots showed that immunoreactive protein was present as a single band at 65,000 Mr in both cell types [6], indicating that the protein was present but it was active in one and silent in the other.

The fact that PKC activation is stereospecific in each target cell supports the contention that the activation of PKC is receptor-mediated. Moreover, treatment of growth zone cells with Ab99 blocks downstream physiological responses of growth zone cells to 1α,25$(OH)_2D_3$ [7], as does inhibition of PKC activity [8]. 1α,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$ may operate via separate receptors but it is also possible that they act via the same receptor through different binding sites, and that this receptor is the tradition nuclear VDR.

To test this hypothesis, we peformed a number of studies using several model systems. Matrix vesicles were isolated from ROS 17/2.8 osteoblast-like cell cultures [2]. Western blots using Ab99 confirmed the presence of the putative 65,000 receptor-associated protein and Ab99 blocked the effect of 1α,25$(OH)_2D_3$ on PKC. Most importantly, no immunoreactive VDR was present on Western

blots. In addition, antibodies to annexin II, which has also been hypothesized to be a membrane-associated receptor for 1α,25(OH)$_2$D$_3$ [9], did not cross-react with the Ab99-positive band in Western blots of the matrix vesicles isolated from the ROS 17/2.8 cell cultures. Anti-annexin II antibodies also did not block the effect of 1α,25(OH)$_2$D$_3$ on PKC in matrix vesicles isolated from growth zone chondrocyte cultures [7].

Although these experiments show that the nuclear VDR was not responsible for the 1α,25(OH)$_2$D$_3$-dependent inhibition of PKCζ in matrix vesicles from growth zone chondrocyte cultures or the 24*R*,25(OH)$_2$D$_3$-dependent inhibition of PKCζ in matrix vesicles from resting zone chondrocyte cultures, they do not rule out the possibility that the VDR plays a role in PKC activation in intact cells. To determine this, we developed technology for culturing costochondral cartilage resting zone and growth zone chondrocytes from wild type and VDR knockout mice [10]. Treatment of these cells with 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ confirmed our previous observation using the rat model. 1α,25(OH)$_2$D$_3$ increased PKC specific activity within 1 min in growth zone cells, with maximal increases at 9 min. 24*R*,25(OH)$_2$D$_3$ increased PKC in resting zone cells within 1 min, with maximal increases at 90 min. As noted in the rat model, PKCα was the responsible isoform. 1α,25(OH)$_2$D$_3$ also caused a rapid increase in PLC specific activity and this effect was only in growth zone cells, indicating that the mechanism in the wild type and VDR knockout mice is the same and like that operating in the rat chondrocytes.

These results show definitively that the nuclear VDR is not responsible for the activation of PKC by 1α,25(OH)$_2$D$_3$ in growth zone cells or for the activation of PKC by 24*R*,25(OH)$_2$D$_3$ in resting zone cells. Moreover, the VDR is not involved in the downstream physiological responses that are mediated by the rapid increase in PKC in the mouse growth zone cells. Treatment of the cells with 1α,25(OH)$_2$D$_3$ in the presence of Ab100, which was generated to the rat homologue of the chick protein described above, blocked the effects of 1α,25(OH)$_2$D$_3$ in the VDR knockout cells. This indicates that the Ab100-reactive protein is important for the membrane-mediated response to 1α,25(OH)$_2$D$_3$.

## 3. Pivotal role of phospholipase A$_2$

1α,25(OH)$_2$D$_3$ activates PKC in growth zone chondrocytes via a PLC-dependent mechanism [11], whereas 24*R*,25(OH)$_2$D$_3$ acts via PLD [12]. Both pathways ultimately result in DAG production but the time course differs. RT-PCR shows that both cell types express mRNAs for the same PLC and PLD isoforms, although basal levels of activity differ. Yet, differences in basal activity are not sufficient to explain why 1α,25(OH)$_2$D$_3$ only activates PLC and only in growth zone cells while 24*R*,25(OH)$_2$D$_3$ only activates PLD and only in resting zone cells.

Studies examining the rapid effects of each metabolite on phospholipid metabolism in growth zone and resting zone cells have provided some clues as to how this differential regulation occurs. 1α,25(OH)$_2$D$_3$ causes a rapid and stereospecific increase in membrane fluidity [13] and arachidonic acid release [14] in growth zone cells, yet has no effect on either parameter in resting zone cells [15]. In contrast, 24*R*,25(OH)$_2$D$_3$ causes a rapid and stereospecific decrease in membrane fluidity [13], and a rapid but short lived decrease in arachidonic acid release [14], in resting zone cells, without altering these parameters in growth zone cells [15]. These observations suggest that the two metabolites regulate PLA$_2$ in a differential manner, since PLA$_2$ catalyzes the release of arachidonic acid. This is also consistent with the fact that resting zone cell membranes and growth zone cell membranes contain a different phospholipid complement [16] and with the fact that 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ differentially regulate the phospholipid composition of the membranes [17]. In addition, 1α,25(OH)$_2$D$_3$ increases PGE$_1$ and PGE$_2$ production by growth zone cells but 24*R*,25(OH)$_2$D$_3$ decreases the production of these prostaglandins by resting zone cells [18].

There is considerable evidence supporting the hypothesis that PLA$_2$ is pivotal in this process. Inhibition of PLA$_2$ blocks the effects of 1α,25(OH)$_2$D$_3$ on growth zone cells [19]. In contrast, activation of the enzyme activity with melittin, which is isolated from bee venom, or mastoparan, which is isolated from snake venom, mimics the effects of 1α,25(OH)$_2$D$_3$ on growth zone cells. The opposite is the case for resting zone cells [20]. Inhibition of PLA$_2$ activates PKC and mimics the effects of 24*R*,25(OH)$_2$D$_3$, whereas activation of PLA$_2$ blocks the stimulatory effect of 24*R*,25(OH)$_2$D$_3$ on PKC.

There are two consequences of PLA$_2$ action on phospholipids: release of archidonic acid and release of lysophospholipids. Exogenous arachidonic acid acts on growth zone cells like 1α,25(OH)$_2$D$_3$ and like PLA$_2$ activation [21]. In contrast, exogenous arachidonic acid acts on resting zone cells in a manner opposite to 24*R*,25(OH)$_2$D$_3$ [20]. Not surprisingly, inhibition of arachidonic acid metabolism with the general cyclooxygenase inhibitor indomethacin causes growth zone cells and resting zone cells to respond to 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ in a manner like their response to exogenous arachidonic acid. Inhibition of inducible cyclooxygenase 2 does not alter that response, whereas inhibition of constitutive cyclooxygenase 1 reduces the arachidonic acid-like effect [22]. This observation shows that PLA$_2$ is rate limiting because it changes substrate concencentration for the consistutive form of the enzyme, and it shows that metabolites of arachidonic acid are also involved in the mechanism.

To better understand how prostaglandin plays a role in the differential activation of PKC by 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$, we examined the regulation of PKC specific activity by PGE$_2$ [23,24]. Growth zone and resting zone cells both express mRNAs for EP1 and EP2, which

are $PGE_2$ receptors. $1\alpha,25(OH)_2D_3$ and $PGE_2$ act via EP1 to activate PKC in growth zone cells and to regulate the physiological responses of these cells that depend on PKC activation. However, $24R,25(OH)_2D_3$ acts through both receptors in resting zone cells, but in unexpected ways. PKC is regulated by $PGE_2$ via EP1 and PKA is regulated via EP2, and both pathways contribute to the physiological respose of resting zone cells to $24R,25(OH)_2D_3$. The regulation is in effect due to de-suppression. By decreasing $PGE_2$ concentration, $24R,25(OH)_2D_3$ reduces the inhibitory effects of arachidonic acid and $PGE_2$ on PKC and PKA, ultimately resulting in the ability of these signaling pathways to generate a MAP kinase response. This explains in part why the maximal effect of $24R,25(OH)_2D_3$ on PKC and MAP kinase in resting zone cells is delayed in comparison with the maximal effects of $1\alpha,25(OH)_2D_3$ in growth zone cells.

We now understand that $PLA_2$ is pivotal in the regulation of PLC as well. The isoform of PLC that is responsible for $1\alpha,25(OH)_2D_3$ action in growth zone cells is phosphatidylinositol-specific PLCβ1 and PLCβ3 [12]. Inhibition of $PLA_2$ blocks $1\alpha,25(OH)_2D_3$-dependent activation of PLC, but this is due to the production of lysophospholipid and not to arachidonic acid. Inhibition of $PLA_2$ has no effect on PLC activity in resting zone cells, presumably in part because lysophospholipid production is reduced to levels that do not support PLC activation. The lack of PI-PLC generated DAG may explain why PKC is not rapidly translocated to the plasma membrane in resting zone cells. Instead, DAG is generated through a PLD-dependent mechanism, which requires two separate catalytic steps and does not result in IP3 production. Consequently, local cytoplasmic increases in DAG may occur more slowly and are not accompanied by rapid release of $Ca^{2+}$ ions from the endoplasmic reticulum that serve as a co-factor in PKCα activation and translocation.

It is relatively easy to understand how activation of $PLA_2$ can positively impact on the activation of PLC and PKC by $1\alpha,25(OH)_2D_3$ in growth zone cells. The studies implicating $PLA_2$ inhibition as a positive regulator of $24R,25(OH)_2D_3$-dependent PKC in resting zone cells are less obvious. As these conclusions relied on experiments that used the bee venom derived $PLA_2$ activator melittin, we repeated them using the rat homologue of melittin, $PLA_2$ activating protein (PLAP). For these experiments, resting zone chondrocytes were isolated from the costochondral cartilage of male Sprague–Dawley rats. Confluent fourth passage cultures were treated with vehicle or PLAP peptide ($10^{-8}$ to $10^{-6}$ M; BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) for 90 min and PKC specific activity determined. PLAP reduced PKC activity in control and $24R,25(OH)_2D_3$-treated cultures in a dose-dependent manner (Fig. 1). In resting zone cells treated with $10^{-6}$ M PLAP, PKC specific activity was reduced by approximately 40%. PLAP ($10^{-6}$ M) completely blocked the stimulatory effect of $10^{-7}$ M $24R,25(OH)_2D_3$ on PKC. PLAP elicited its effects on resting zone cells by activating $PLA_2$. Inhibition of $PLA_2$ activity with quinacrine caused a dose-dependent

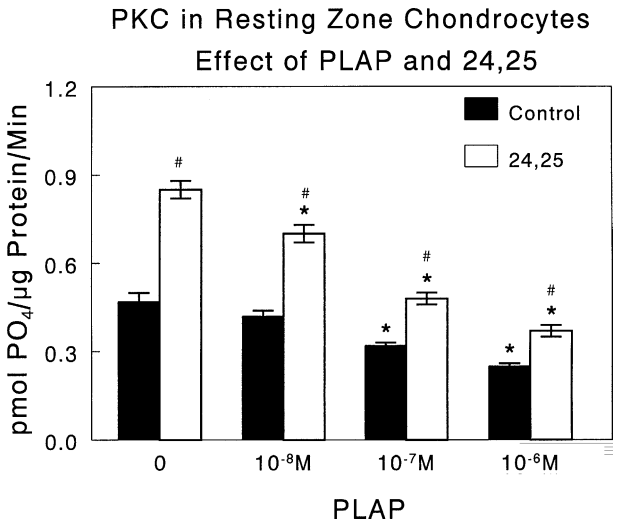


Fig. 1. Effect of $PLA_2$ activating protein (PLAP) on PKC specific activity in resting zone cells treated with $24R,25(OH)_2D_3$. Confluent cultures of rat costochondral resting zone chondrocytes were treated with vehicle or $10^{-7}$ M $24R,25(OH)_2D_3$ (24,25) for 90 min. One-half of the cultures were also treated with PLAP peptide (0, $10^{-8}$, $10^{-7}$, or $10^{-6}$ M). PKC activity was determined in cell layer lysates using an immunoassay kit. Results were normalized to cell layer protein content. Values are means ± SEM for six independent cultures for each variable. Data were analyzed by ANOVA and specific differences between groups determined using the Bonferroni modification of Student's *t*-test. $^{*}P < 0.05$, with PLAP vs. without PLAP; $^{\#}P < 0.05$, with $24R,25(OH)_2D_3$ vs. without $24R,25(OH)_2D_3$ at each PLAP concentration.

increase in PKC activity of control cultures and reversed the inhibitory effects of PLAP (Fig. 2). This was due to the release of arachidonic acid and not to metabolites of the fatty acid produced as a consequence of the action of cyclooxygenase. Treatment of the cells with $10^{-8}$ to $10^{-6}$ M indomethacin caused a dose dependent increase in PKC activity in control cultures and reversed the inhibition of PKC due to PLAP (Fig. 3). PLAP had no effect on the increase in PLD activity due to $24R,25(OH)_2D_3$ in resting zone cells (Fig. 4A), indicating that $PLA_2$ activation does not mediate the effects of $24R,25(OH)_2D_3$ on this enzyme. In addition, stimulation of resting zone cells with exogenous PLAP did not confer sensitivity to $1\alpha,25(OH)_2D_3$ to the resting zone cells, at least over the 90 min incubation period used in this study (Fig. 4B).

PLAP is not expressed in resting zone cells in vivo, either in the form of mRNA (in situ hybridization), or in the form of protein (immunohistochemistry) (data not shown); thus, activation of $PLA_2$ and consequent inhibition of PKC is not physiologically relevant in these cells. However, the absence of PLAP argues for the physiological relevance of $PLA_2$ inhibition by $24R,25(OH)_2D_3$ in the activation of PKC in resting zone cells. As is the case for $PLA_2$ specific activity [25], constitutive levels of PLC specific activity are higher in resting zone cells cells [12]. By inhibiting $PLA_2$ activity, $24R,25(OH)_2D_3$, may reduce production of lysophopholipids and by so doing, depress PLC activity

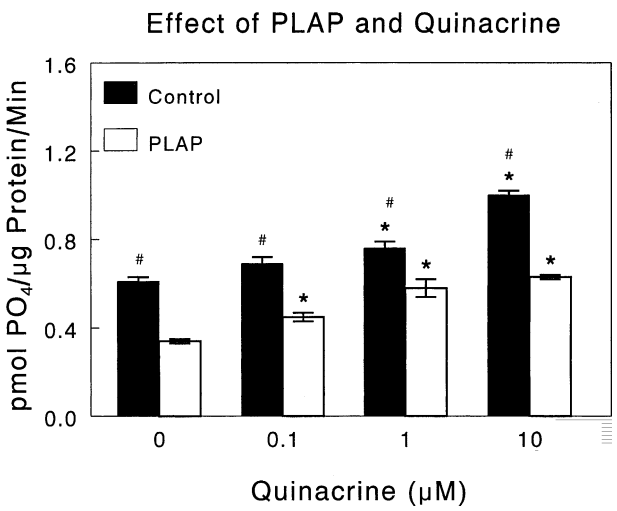


Fig. 2. Effect of $PLA_2$ inhibition on PLAP peptide dependent inhibition of PKC specific activity in rat costochondrdal resting zone cartilage cells. Confluent cultures of resting zone cells were treated for 90 min with control media or media containing $10^{-6}$ M PLAP. One-half of the cultures were also treated with 0, 0.1, 1 or 10 μM quinacrine to inhibit $PLA_2$ activity. PKC activity was determined in cell layer lysates using an immunoassay kit. Results were normalized to cell layer protein content. Values are means ± SEM for six independent cultures for each variable. Data were analyzed by ANOVA and specific differences between groups determined using the Bonferroni modification of Student's *t*-test. $^{*}P < 0.05$, with quinacrine vs. without quinacrine; $^{\#}P < 0.05$, with PLAP vs. without PLAP at each quinacrine concentration.

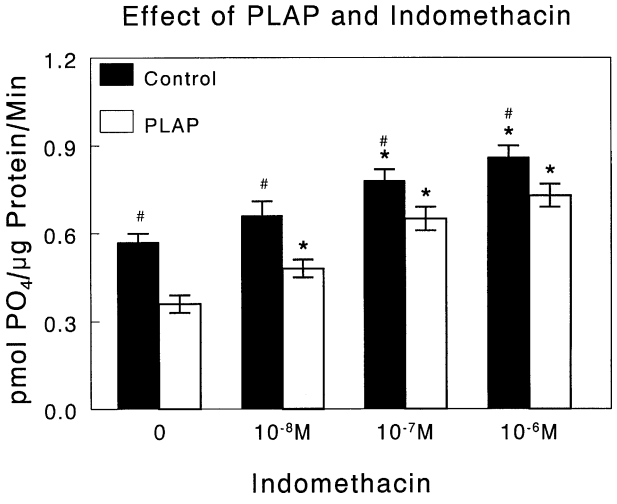


Fig. 3. Effect of indomethacin on the PLAP peptide dependent inhibition of PKC specific activity in rat costochondral resting zone cartilage cells. Confluent cultures of resting zone cells were treated for 90 min with control media or media containing $10^{-6}$ M PLAP. One-half of the cultures were also treated with 0, $10^{-8}$, $10^{-7}$, or $10^{-6}$ M indomethacin to inhibit cyclooxygenase activity. PKC activity was determined in cell layer lysates using an immunoassay kit. Results were normalized to cell layer protein content. Values are means ± SEM for six independent cultures for each variable. Data were analyzed by ANOVA and specific differences between groups determined using the Bonferroni modification of Student's *t*-test. $^{*}P < 0.05$, with indomethacin vs. without indomethacin; $^{\#}P < 0.05$, with PLAP vs. without PLAP at each indomethacin concentration.

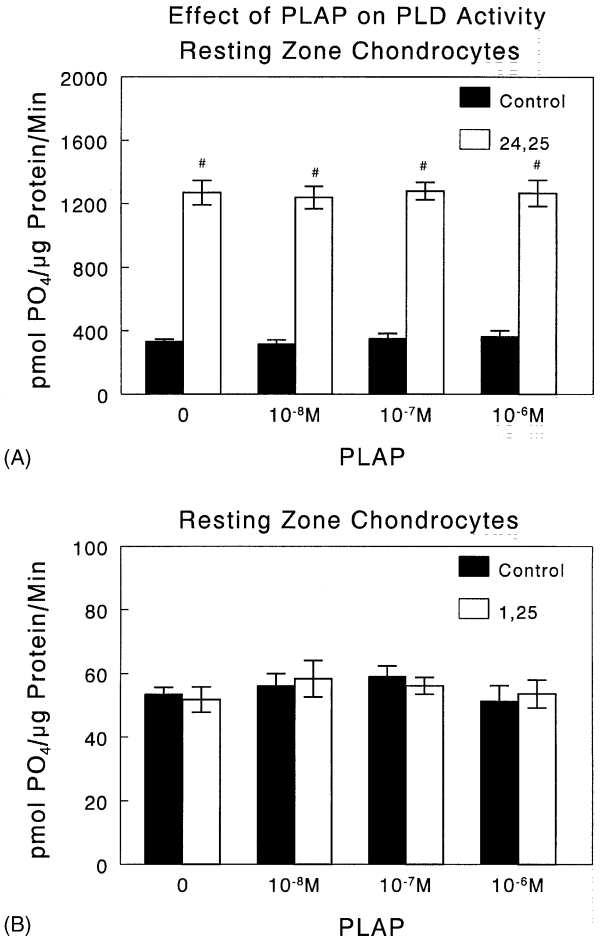


Fig. 4. Effect of PLAP peptide on phospholipase D (PLD) specific activity in resting zone cells treated with 24*R*,25$(OH)_2D_3$. Confluent cultures of rat costochondral resting zone chondrocytes were treated with vehicle or $10^{-7}$ M 24*R*,25$(OH)_2D_3$ (24,25) (Panel A) or $10^{-8}$ M 1α,25$(OH)_2D_3$ (1a,25) (Panel B) for 90 min. One-half of the cultures were also treated with PLAP peptide (0, $10^{-8}$, $10^{-7}$, or $10^{-6}$ M). PLD activity was determined in cell layer lysates using an kit. Results were normalized to cell layer protein content. Values are means ± SEM for six independent cultures for each variable. Data were analyzed by ANOVA and specific differences between groups determined using the Bonferroni modification of Student's *t*-test. 24*R*,25$(OH)_2D_3$ caused a statistically significant increase in PLD specific activity. No other differences were found.

in the resting zone cells. This would then explain why PLD-dependent DAG is required for PKC activity rather than the more rapidly produced PLC-dependent DAG associated with 1α,25$(OH)_2D_3$-stimulated PKC signaling [12]. A similar line of reasoning would explain the inhibitory effects of arachidonic acid and $PGE_2$ on PKC in resting zone cells whereas both phospholipid metabolites are stimulatory in growth zone cells [1].

## 4. Summary

These studies demonstrate the critical and pivotal role that $PLA_2$ plays in the mechanism of action of both Vitamin D

metabolites in cartilage. Others have shown that $PLA_2$ is important to the action of $1\alpha,25(OH)_2D_3$ in smooth muscle cells [26] and have suggested a role for $PLA_1$ as well. Signaling pathways initiated by this rapid response mediate some but not all of the physiological responses of the cells to $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$. The results shown here indicate that the effects of $24R,25(OH)_2D_3$ on resting zone cells are replicated by exogenous PLAP, indicating that the $PLA_2$ dependent pathways are involved and suggesting that PLAP may play a role in growth zone cells as well. More recent studies from our laboratory show that events initiated by the membrane receptor cooperate with events regulated via the tranditional nuclear Vitamin D receptor. MAP kinase is a critical component of this interaction [5], as has been noted in other systems [27,28].

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