THE ROLE OF PHOSPHOLIPASE A_2 AND THE PRODUCTS OF ITS ACTION IN THE REGULATION OF CHONDROCYTES BY VITAMIN D_3 METABOLITES

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DEDICATION

I dedicate this thesis to my mother, Janet L. Curry, and to the loving memory of my father, LeRoy R. Curry. Their lifelong love and support have allowed me to achieve my desired professional and personal goals. To them I say thank you!

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THE ROLE OF PHOSPHOLIPASE A_2 AND THE PRODUCTS OF ITS ACTION IN THE REGULATION OF CHONDROCYTES BY VITAMIN D_3 METABOLITES

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Long bone formation, osseous repair, and potentially, guided tissue regeneration, all occur through endochondral bone formation, a process which involves chondrocyte differentiation, proliferation, hypertrophy, and mineralization. Vitamin D_3 metabolites have been shown to play a pivotal role in chondrocyte differentiation and are therefore essential for proper endochondral ossification. In fact, the actions of the vitamin D_3 metabolites are cell maturation-dependent with 1,25-$(OH)_2D_3$ primarily affecting growth zone chondrocytes and 24,25-$(OH)_2D_3$ primarily affecting resting zone chondrocytes. Prior studies have demonstrated that 1,25-$(OH)_2D_3$

increases phospholipase A_2 activity in growth zone chondrocytes, while 24,25-$(OH)_2D_3$ decreases phospholipase A_2 activity in resting zone chondrocytes. In addition, these vitamin D_3 metabolites mediate their effects in a cell maturation-dependent manner through activation of protein kinase C (PKC), an enzyme involved in cellular signal transduction. The aim of the present study was to examine the role of phospholipase A_2 in mediating the activation of PKC by vitamin D_3 metabolites.

Resting zone and growth zone cells were cultured in the presence or absence of various phospholipase A_2 activators (melittin or mastoparan), phospholipase A_2 inhibitors (quinacrine, OEPC [secretory phospholipase A_2], or $AACOCF_3$ [cytosolic phospholipase A_2]), exogenous arachidonic acid, prostaglandin E_2, or indomethacin (an inhibitor of cyclooxygenase) alone or in the presence of the target cell specific vitamin D_3 metabolites. The protein content in each sample was determined using a colorimetric assay while the PKC specific activity in the cell layer was assessed by a standard filter binding assay measuring the incorporation of ^{32}P into myelin basic protein.

In resting zone chondrocytes, inhibition of phospholipase A_2 activity resulted in a dose-dependent increase in the level of PKC in both control and 24,25-$(OH)_2D_3$-treated cultures. By contrast, the addition of the phospholipase A_2 inhibitors to growth zone cell cultures resulted in a dose-dependent decrease in PKC in both control and 1,25-$(OH)_2D_3$-treated cultures. When the phospholipase A_2 activators, melittin or mastoparan, were added to the cultures, basal PKC specific activity in resting zone cells was reduced while that in growth zone cells was increased, both in a dose- and time-dependent manner. Similarly, melittin and mastoparan decreased 24,25-$(OH)_2D_3$-stimulated PKC specific activity in resting zone chondrocytes and increased 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone chondrocytes. With both cell types, the

addition of arachidonic acid to the culture media produced an effect on PKC specific activity that was again dose- and time-dependent and nearly identical to that observed following the addition of the phospholipase A_2 activators. Furthermore, when prostaglandin E_2, a downstream product of arachidonic acid metabolism via the cyclooxygenase pathway, was added to the cell cultures, changes in PKC specific activity displayed dose-dependent trends similar to those observed following the addition of the phospholipase A_2 activators or arachidonic acid. Moreover, when prostaglandin E_2 production was blocked with indomethacin, a cyclooxygenase inhibitor, the result was again cell-maturation and dose-dependent. While the addition of indomethacin to resting zone cell cultures resulted in a dose-dependent stimulation of the 24,25-$(OH)_2D_3$-induced activation of PKC specific activity, the opposite effect was observed in growth zone chondrocytes where it resulted in a dose-dependent reduction of the 1,25-$(OH)_2D_3$-induced PKC specific activity.

These results demonstrate that the effect of 24,25-$(OH)_2D_3$ on PKC specific activity in resting zone cells and that of 1,25-$(OH)_2D_3$ in growth zone cells is mediated by changes in phospholipase A_2 activity, release of arachidonic acid, and its major metabolite, prostaglandin E_2. The effect is time-, dose-, and cell maturation-dependent and metabolite-specific. Our research indicates that it is not only possible to stimulate PKC specific activity with vitamin D_3, but also to enhance the activity of this enzyme by adding various activators or inhibitors of phospholipase A_2 activity. The potential to selectively stimulate or inhibit PKC specific activity in chondrocytes provides a rational approach for ultimately regulating bone and cartilage formation. Consequently, this information may be used to enhance mineralization and potentially lead to an improvement in the predictability of periodontal or bone regeneration.

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INTRODUCTION AND LITERATURE REVIEW

A. Endochondral Bone Formation

Long bone formation, osseous repair, and guided tissue regeneration (Amar et al., 1997) occur through endochondral bone formation, a process which involves chondrocyte differentiation, proliferation, hypertrophy, and mineralization. Endochondral ossification involves several cell types including resting zone chondrocytes, growth zone chondrocytes, osteoclasts, and osteoblasts. The activity of these cells is directed by regulatory factors such as vitamin D_3 metabolites ($1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$), parathyroid hormone, growth factors, prostaglandins, interleukins, tumor necrosis factor-α, glucocorticoids, and bone morphogenetic proteins (Boyan et al., 1997a; Cochran and Wozney, 1999).

For successful endochondral ossification, cells progress through a series of maturational stages as they undergo their lineage cascade. Initially, mesenchymal stem cells are induced to become osteochondral progenitor cells, then chondroprogenitor cells, and finally, chondroblasts (Boyan et al., 1997a). Once the cells are committed to the chondrogenic pathway, their further maturation is limited to this lineage (Caplan, 1991; Beresford, 1989; Owen, 1985; Fridenstein, 1990). The earliest chondrocytic cell formed is the resting zone chondrocyte, an immature cell involved in the production and maintenance of a proteoglycan-rich type II collagen matrix (Solursh et al., 1978; Schmid et al., 1985; Stocum et al., 1979; Pacifici et al., 1991). The length of time a cell spends at this stage of maturation is unknown. By mechanisms that are not clearly understood, hormonal signals stimulate the cells to undergo a proliferative burst and differentiate into growth zone chondrocytes (prehypertrophic and hypertrophic cells). These cells increase markedly in size and prepare their matrix for mineralization by changing the composition of their

extracellular matrix (Dean et al., 1985). The growth zone cells then produce type X collagen (Schmid et al., 1985; Pacifici et al., 1991; Kielty et al., 1985) as well as proteins normally associated with bone and mineralization, namely osteocalcin, osteopontin, and type I collagen (Gerstenfeld et al., 1990a; Gerstenfeld et al., 1990b; Quarto et al, 1993). Once the matrix is prepared, mineralization occurs through hydroxyapatite formation. Due to the avascular nature of cartilage, the mineralization process prevents cells from receiving nutrients through diffusion and subsequently cellular death occurs via apoptosis (Roach, 1995). Osteoclasts are then activated to resorb the cells as well as their calcified matrices. Coupled with this osteoclastic activity, however, is osteoblastic activity and vascular invasion. Osteoprogenitor cells migrate to the calcified cartilage scaffold and bone formation begins (Pechak et al., 1986a). The process of endochondral ossification is complete once the cartilage within the newly formed bone is resorbed and a marrow cavity formed (Pechak et al., 1986a; Pechak et al., 1986b; Caplan and Pechak, 1987).

B. The Significance of Cartilage

Orthopaedic research pertaining to mineralizing tissues has traditionally utilized cartilage as a type of model system. This system takes advantage of the fact that bone and cartilage respond similarly to several growth factors and vitamin D_3 metabolites (Boyan et al., 1988; Saggese et al., 1993; Andrew et al., 1993; Chiba and Matsuyama, 1993). Other findings linking the two tissues include reports that following apoptosis of the growth zone chondrocytes, certain sister cells survive and form osteoblasts (Roach et al., 1995; Roach, 1992). In addition, it has been suggested that certain cartilaginous cells may "transdifferentiate" into osteoblasts in the mandibular condyle (Beck et al., 1991; Thesingh et al., 1991). The benefit of using a cartilaginous model is that it allows for the independent evaluation of specific effects on cells, to

be based on the level of cell maturation. Thus, the complex phenomenon known as endochondral ossification can be more easily evaluated.

Cartilage serves as an appropriate model not only for orthopaedic research, but also periodontal research. Although the mandible and maxilla are both formed embryologically through intramembranous bone formation, substantial evidence suggests endochondral bone formation is involved in the subsequent regulation of the periodontium. In particular, it has been demonstrated that the osseous repair of intramembranous bones is similar to endochondral bone formation (Girgis and Pritchard, 1958; Hall and Jacobson, 1975). In fact, when demineralized bone matrix is implanted subcutaneously, bone is formed ectopically via endochondral bone formation (Reddi and Anderson, 1976; Zhang et al., 1997). Finally, recent histological evidence suggests guided tissue regeneration also proceeds through the endochondral pathway (Amar et al., 1997).

C. The Rat Costochondral Chondrocyte Model

To study the process of endochondral ossification, several models have been used. Comparable histological and cytological results in endochondral bone formation have been found using chickens (Wuthier, 1982), rats (Boyan et al., 1988), rabbits (Takigawa et al., 1988), or bovines (Ray et al., 1982). Cartilage from chick and rat growth plate have been the most widely used for studying chondrocytes in long-term culture. The growth plate in the chick is arranged with a linear alignment of chondrocytes with the least mature cells (resting zone) at the top and the most mature cells (growth zone) at the bottom. The culture process initially involves the separation of cell zones by sharp dissection, and subsequent, cellular release by collagenase digestion. The problem with the chick growth plate model is cross-contamination of cell types (Boyan et al., 1997a). Because the growth plate is irregular and highly interdigitated with bone

spicules, bone cell contamination is a legitimate concern. To overcome this possibility, the rat costochondral chondrocyte model was developed (Boyan et al., 1988). In this model, the chondrocytes are also arranged in a linear array with increasing cell-maturation as the mineralization front is approached. However, the interdigitation of bone is not present. Using sharp dissection, the resting zone (reserve zone) cartilage can be separated from the adjacent bone and proliferative cartilage while the prehypertrophic/upper hypertrophic zone (growth zone) can be separated from the proliferative zone and calcified cartilage. To ensure a clean demarcation of cell maturation, only cultures of reserve zone and growth zone cells are made. To increase cell number without loosing expression of phenotypic markers, cells are cultured through the fourth passage (Schwartz et al., 1989). The clean dissection of resting zone cells from growth zone cells allows for the independent evaluation of effects based on maturation state. In particular, the differential responsiveness to factors including vitamin D are possible.

D. Vitamin D Metabolites

Vitamin D plays an important regulatory role in chondrocyte differentiation and maturation (Atkin et al., 1985; Binderman and Somjen, 1984) and therefore is essential for proper endochondral ossification (Boskey, 1981; Anderson, 1969). Vitamin D is not a true vitamin, but rather a secosteroid which is synthesized in the skin from 7-dehydrocholesterol after exposure to ultraviolet irradiation. Vitamin D is also found in certain foods and is used to supplement dairy products. Both cholecalciferol (vitamin D_3), the natural form, and ergocalciferol (vitamin D_2), the plant-derived form, are present in the diet. Cholecalciferol is the predominant form of vitamin D, however, if sunlight is lacking, ergocalciferol in the diet is critical in the prevention of rickets. Both forms of vitamin D are biologically inactive when they enter the blood stream and are initially hydroxylated in the liver to 25-hydroxycholecalciferol [25-(OH)D_3], the main circulating

form of vitamin D. This metabolite undergoes further hydroxylation in the kidney to 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] or 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$]. In addition to the production of vitamin D in the liver and kidneys, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ are produced in the growth plate by both resting zone and growth zone chondrocytes (Schwartz et al., 1992b).

The functions associated with vitamin D_3 metabolites are distinct and metabolite- specific. Although both metabolites are involved in chondrogenesis, 24,25-$(OH)_2D_3$ is specific for the initiation of chondrogenesis and 1,25-$(OH)_2D_3$ for calcification. 24,25-$(OH)_2D_3$ regulates the synthesis of DNA, proteoglycan, and type II collagen (Corvol et al., 1978; Corvol et al., 1980). These cellular activities are associated with inducing chondrocyte differentiation and maturation and inhibiting proliferation (de Boland et al., 1992; Atkin et al., 1985). On the other hand, 1,25-$(OH)_2D_3$ promotes Ca^{2+} transport (Lieberherr et al., 1989), maintains extracellular Ca^{2+} concentrations, and regulates calcified cartilage differentiation and maturation (Norman, 1980). These regulatory roles are possible because 1,25-$(OH)_2D_3$ functions to increase serum Ca^{2+} and phosphate levels by stimulating absorption in the small intestine while inhibiting excretion from the kidney.

The specificity of 24,25-$(OH)_2D_3$ for initiation of chondrogenesis and of 1,25-$(OH)_2D_3$ for calcification is observed *in vivo*. In a study of ectopic bone formation, the levels of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were followed after implantation of DFDBA (Weintroub et al., 1982). An increase in the concentration of 24,25-$(OH)_2D_3$ coincided with chondrogenesis, whereas the concentration of 1,25-$(OH)_2D_3$ reached maximal levels during bone formation and subsequent remodeling. A similar trend was observed in chick fracture callus formation (Lidor et al., 1987).

In vitro studies evaluating vitamin D_3 metabolite production by growth plate chondrocytes reveal a similar pattern (Schwartz et al., 1992b). The fact that the more highly differentiated growth zone chondrocytes exhibited greater production of 1,25-$(OH)_2D_3$ and that this production was stimulated by 24,25-$(OH)_2D_3$ indicates that 1,25-$(OH)_2D_3$ regulates the more mature cartilage zone, and 24,25-$(OH)_2D_3$ regulates the less differentiated resting zone chondrocytes (Schwartz et al., 1992b; Schwartz et al., 1995). It further suggests that 1,25-$(OH)_2D_3$ specifically affects the growth zone chondrocytes to prepare them for mineralization and that 24,25-$(OH)_2D_3$ regulates the maturation cascade of the resting zone chondrocytes.

Although the metabolites exert different effects on growth plate cartilage by targeting different cells, the regulation of endochondral bone formation requires both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ (Boyan et al., 1997a). Autoradiographic evidence suggests receptors for both metabolites exist in the growth plate, with certain subpopulations of cells responding in a metabolite-specific manner (Corvol et al., 1980; Fine et al., 1985; Balmain et al., 1993). In particular, putative receptors for 24,25-$(OH)_2D_3$ are localized to the proliferating cells in the growth plate, whereas those for 1,25-$(OH)_2D_3$ are found in osteoprogenitor cells and osteoblasts. Consistent with these observations is the fact that the chondrocyte response to both 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ is cell maturation-dependent.

In general, resting zone chondrocytes respond primarily to 24,25-$(OH)_2D_3$ while growth zone chondrocytes respond primarily to 1,25-$(OH)_2D_3$ (Boyan et al., 1988). Maturation-dependent effects are evidenced by differences in extracellular protein synthesis (Schwartz et al., 1989), matrix vesicle and plasma membrane enzyme activities (Dean et al., 1996; Boyan et al., 1994; Dean et al., 1992; Schwartz and Boyan, 1988a; Schwartz et al., 1988b), cell proliferation (Schwartz et al., 1989), arachidonic acid turnover (Schwartz et al., 1990; Swain et al., 1992),

prostaglandin production (Schwartz et al., 1992a), Ca^{2+} flux (Langston et al., 1990), vitamin D metabolite production (Schwartz et al., 1992b), and protein kinase C specific activity (PKC) (Sylvia et al., 1993). In particular, 1,25-$(OH)_2D_3$ treatment of growth zone chondrocyte cultures increases alkaline phosphatase (Schwartz et al., 1988b), phospholipase A_2 activity (Schwartz and Boyan, 1988a; Schwartz et al., 1988b), and prostaglandin E_2 production (Schwartz et al., 1992a). By contrast, 24,25-$(OH)_2D_3$ treatment of resting zone chondrocyte cultures increases alkaline phosphatase (Schwartz et al., 1988b) while decreasing both phospholipase A_2 activity (Schwartz and Boyan, 1988a; Schwartz et al., 1988b) and prostaglandin E_2 production (Schwartz et al., 1992a). In addition, 24,25-$(OH)_2D_3$ regulates the differentiation and maturation of the resting zone chondrocytes into growth zone chondrocytes (Schwartz et al., 1995). Finally, another early target cell specific response to vitamin D_3 metabolites is an increase in PKC specific activity (Berry et al., 1996; Sylvia et al., 1993). In growth plate cartilage, the increase in PKC specific activity is metabolite-specific and cell maturation-dependent. 1,25-$(OH)_2D_3$ stimulates PKC specific activity in growth zone cell cultures and 24,25-$(OH)_2D_3$ stimulates PKC specific activity in resting zone cell cultures (Sylvia et al., 1993; Sylvia et al., 1996).

E. Protein Kinase C

There are a number of mechanisms used by cells to respond to signals (cytokines, hormones, growth factors) from the extracellular environment. In general, the extracellular ligands can either bind to receptors on the cellular membrane or directly traverse the membrane. Once associated with the membrane, the ligands activate transducers such as G-proteins at the receptor site which in turn activate intracellular effectors either directly or through various kinases. Examples of these effectors are adenylate cyclase, phospholipase A_2, phospholipase C, and phospholipase D. Second messengers such as cyclic AMP, 1,2-diacylglycerol (DAG), inositol

1,4,5-trisphosphate, and Ca^{2+} are activated by effectors and then increase the activity of target proteins including protein kinase C and protein kinase A. The target proteins can either act on transducers or receptors to turn off signal transduction (Karin, 1992) or allow for continued intracellular signaling by regulating other proteins, ultimately resulting in gene expression.

PKC is a large family of isoenzymes which phosphorylate protein substrates on serine or threonine residues (Parker and Dekker, 1997). There are two main categories of PKC; a Ca^{2+} dependent conventional group (cPKC) which contains the isoenzymes α, β_1, β_2, and γ; and a Ca^{2+} independent novel group (nPKC) which contains the isoenzymes δ, ε, ξ, η, and θ (Hug and Sarre, 1993). All members of the cPKC group are phospholipid dependent while all members of the nPKC group are phospholipid independent except isoenzymes δ and ε.

Depending on the isoform stimulated, PKC specific activity can result in either catabolic or anabolic effects. For example, lipopolysaccharide from putative periodontal pathogens is known to activate PKC isoform ε, ultimately resulting in the destruction of the periodontium. This inflammatory response is a result of lipopolysaccharide-stimulated macrophage secretion of tumor necrosis factor α, interleukin-1, and nitric oxide mediated through PKC (Shapira et al., 1997). By contrast, PKCα is the major isoform in cell layer lysates and plasma membranes of both resting zone and growth zone cells isolated from rat costochondral cartilage (Sylvia et al., 1996). In these cells, PKCα stimulation results in activities associated with endochondral bone formation.

The diverse role that PKC plays in directing cellular signal transduction has only recently begun to emerge. PKC dictates cellular activation, growth, differentiation and mineralization by mediating the transduction of extracellular signals to intracellular effectors (Ohno et al., 1991; Inoue et al., 1977). PKC accomplishes this by playing a pivotal role in the integration of various phospholipase signaling pathways (Liscovitch, 1992), three of which are phospholipase A_2,

phospholipase C, and phospholipase D. The phospholipases are important because they hydrolyze membrane phospholipids and generate a variety of second messenger molecules. For example, phospholipase A_2 acts on the *sn*-2 position of membrane phospholipids to generate free arachidonic acid, the rate-limiting precursor for the synthesis of prostaglandins and leukotrienes (Nakamura et al., 1994). In addition, phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate the second messengers DAG and inositol 1,4,5-trisphosphate. DAG is extremely important because it is an allosteric activator of PKC (Hug and Sarre, 1993; Liyanage et al., 1992). Certain forms of phospholipase C can also generate DAG by hydrolyzing the principle membrane phospholipid, phosphatidylcholine (McAllister et al., 1994). Similar studies in osteoblasts have also supported the role of phospholipase C in the activation of PKC via DAG (Civitelli et al., 1990). Finally, phospholipase D also plays an important role by cleaving phosphatidylcholine into phosphatidic acid and choline. Phosphatidic acid can then be dephosphorylated into DAG, again resulting in activation of PKC.

The importance of DAG as a prerequisite for PKC activation and cell response cannot be overstated. Sustained elevations of DAG are required for long-term responses such as cell growth and differentiation (Asaoka et al., 1992). Initially high levels of DAG disappear rapidly. After a lag period, however, DAG frequently increases again and persists longer, occasionally for several hours (Nakamura et al., 1994). The cause of the discrepancy in DAG levels was resolved once the mechanisms responsible for DAG stimulation were defined. Research demonstrated that the initially high levels of DAG were associated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate via phospholipase C while the delayed and sustained DAG levels were associated with the hydrolysis of phosphatidylcholine via phospholipase C or phospholipase D (Nakamura et al., 1994; McAllister et al., 1994).

To understand the maturation- and metabolite-specific manner in which PKC is stimulated in rat costochondral chondrocytes, it is imperative to analyze differences in the potential regulatory pathways. Previously, it has been demonstrated that PKC specific activity in growth zone chondrocyte cultures is increased following treatment with 1,25-$(OH)_2D_3$ with maximal PKC stimulation occurring at 9 minutes after treatment. By contrast, PKC specific activity in resting zone chondrocyte cultures treated with 24,25-$(OH)_2D_3$ peaks at 90 minutes (Sylvia et al., 1993). The cell maturation- and metabolite-specific effect on PKC specific activity is also observed if phospholipase C is inhibited with U73122. While this inhibitor decreased PKC specific activity in growth zone cell cultures stimulated with 1,25-$(OH)_2D_3$, it had no effect on PKC specific activity in resting zone cell cultures stimulated with 24,25-$(OH)_2D_3$ (Helm et al., 1996). Additionally, neither actinomycin D (a transcription inhibitor) nor cycloheximide (a translation inhibitor) affected 1,25-$(OH)_2D_3$-induced PKC specific activity in growth zone chondrocyte cultures, while both inhibitors blocked 24,25-$(OH)_2D_3$-induced PKC specific activity in resting zone chondrocyte cultures (Sylvia et al., 1993). As evidenced by the rapid stimulation of PKC specific activity in growth zone chondrocytes, the effects of 1,25-$(OH)_2D_3$ appear to be nongenomic and at least partially modulated via phospholipase C stimulation, independent of tyrosine kinase (Helm et al., 1996). By comparison, the effect of 24,25-$(OH)_2D_3$ on PKC specific activity in resting zone cells appears to be at least partially genomic (Sylvia et al., 1993). A nongenomic action of the hormone is one that does not require new gene transcription or protein synthesis and it is normally quicker in effect than its genomic counterpart.

F. Phospholipase A_2, Arachidonic Acid, and Prostaglandin E_2

The major phospholipase activity in mineralizing cartilage is phospholipase A_2 (Rasmussen et al., 1982) and this activity is positively correlated with the differentiation of the epiphyseal

growth plate (Wuthier, 1973). Several pro-inflammatory factors, such as tumor necrosis factor, interleukin-1 and lipopolysaccharide have been shown to stimulate expression and secretion of phospholipase A_2 (Piomelli, 1993). Activation of phospholipase A_2 is recognized as the rate limiting step in the release of arachidonic acid and the subsequent conversion into a family of biologically active metabolites, eicosanoids (Bomalaski and Clark, 1993; Bell et al., 1979).

Arachidonic acid is the primary polyunsaturated fatty acid released from membrane phospholipids. Release occurs by two major pathways; cleavage of the glycerophospholipid backbone at the *sn*-2 position, or DAG hydrolysis by DAG-lipase (Piomelli, 1993). The free arachidonic acid is subsequently metabolized by one of three pathways. The cytochrome P-450 pathway catalyzes the conversion of arachidonic acid into hydroxyacids (HETE) and diols while the lipoxygenase pathway leads to the formation of leukotrienes, epoxyhydroxides, and HETE. The third pathway involves the enzymatic conversion of arachidonic acid to prostaglandins, prostacyclins and thromboxanes via the cyclooxygenase pathway.

Prostaglandins are 20-carbon unsaturated fatty acids that contain a cyclopentane ring (Trummel, 1980). There are 9 groups of prostaglandins which are designated as A-I according to the structural variations of the cyclopentane ring. In addition, each prostaglandin except prostaglandin I (PGI) can be classified in one of three series, designated by a subscript 1, 2 or 3 following the group designation. This subscript indicates the total number of double bonds in the 2 side chains of the prostaglandin molecule (i.e. PGE_2 has 2 double bonds). Prostaglandins are produced primarily from cells of monocyte/macrophage lineage (Dy et al., 1980) although many other cells, including osteoblasts (Schwartz et al., 1992c), maintain the ability to synthesize these mediators upon stimulation (Van Dorp et al., 1974). Once released, they act on designated

tissues via specific receptors before ultimately undergoing rapid metabolism in the liver, lungs, and other tissues (Trummel, 1980).

The biological effects of prostaglandins are extremely diverse and short-lived. Not only are slight variations in prostaglandin structure accompanied by profound differences in biological action, but the same prostaglandin may exert opposite effects in different tissues or at different concentrations. As an example, depending on the concentration of prostaglandin E_2, the effect can be either catabolic or anabolic. High serum and tissue levels inhibit bone collagen synthesis (Raisz, 1974) as well as promote acute and chronic inflammation. In terms of periodontal disease, there is an increasing body of evidence which correlates high concentrations of prostaglandin E_2 with the destruction of the periodontium (Goodson et al., 1974; Offenbacher et al., 1986; Ohm et al., 1984). Lower concentrations, on the other hand, stimulate both DNA and collagen synthesis and promote bone matrix synthesis (Chyun and Raisz, 1984). In fact, when prostaglandin E_2 production is inhibited with nonsteroidal anti-inflammatory drugs, there is a significant reduction in attachment loss and alveolar bone resorption (Williams et al., 1989; Williams et al., 1991). The dual role played by prostaglandin E_2 suggests an important regulatory role in endochondral ossification.

G. Purpose of Investigation

In growth zone chondrocytes, 1,25-$(OH)_2D_3$ elicits rapid actions including changes in calcium ion flux (Farach-Carson et al., 1991, Norman et al., 1994; Langston et al., 1990), arachidonic acid turnover (Schwartz et al., 1990), and membrane fluidity (Swain et al., 1993). In addition, PKC specific activity is increased (Sylvia et al., 1993; Berry et al., 1996) without new gene expression, G-protein activation (Sylvia et al., 1998), or tyrosine kinase activity (Helm et al., 1996). However, the pathways through which this is accomplished remain ambiguous. It has

been previously reported that 1,25-$(OH)_2D_3$ specifically increases PKCα (Sylvia et al., 1993), the Ca^{2+}- and phospholipid-dependent isoform. This suggests that 1,25-$(OH)_2D_3$-induced activity in growth zone chondrocytes may occur through changes in phospholipid metabolism. In fact, phospholipase C has been shown to play an important role by increasing the production of DAG, an allosteric activator of PKC (McAllister et al., 1994). Additionally, recent investigations have demonstrated that a specific cytosolic phospholipase A_2 inhibitor caused a reduction in both basal- and 1,25-$(OH)_2D_3$-induced PKC specific activity (Boyan et al., 1997b).

In resting zone chondrocytes, 24,25-$(OH)_2D_3$ also elicits similar membrane-mediated effects including changes in calcium ion flux (Langston et al., 1990), arachidonic acid turnover (Schwartz et al., 1990), and membrane fluidity (Swain et al., 1993). Although PKCα is also stimulated by 24,25-$(OH)_2D_3$ (Sylvia et al., 1993), the mechanism appears to be different from that seen in the more mature growth zone chondrocytes. Phospholipase C is not involved, the time coarse is not as rapid, and there is new gene expression. In the absence of phospholipase C, PKCα stimulation may involve both phospholipase A_2 and phospholipase D. Studies utilizing specific inhibitors of phospholipase A_2 have demonstrated that decreased phospholipase A_2 activity caused an increase in both basal- and 24,25-$(OH)_2D_3$-induced PKC specific activity (Helm et al., 1996). In addition, a role for phospholipase D is suggested through increased DAG production independent of phospholipase C (Helm et al., 1996).

Increased DAG production, whether achieved through phospholipase C or phospholipase D activity, however, does not explain the specificity of the PKC response to the vitamin D_3 metabolites. Regardless of the state of endochondral maturation, there is a net increase in DAG and PKC specific activity in both cell types. The fact that 1,25-$(OH)_2D_3$ stimulates phospholipase A_2 activity, whereas 24,25-$(OH)_2D_3$ inhibits this activity (Swain et al., 1992), suggests that this

enzyme may partially account for the observed differential regulation of PKC specific activity in growth zone and resting zone chondrocytes. *Therefore, the purpose of the present study was to examine the hypothesis that PKC specific activity is regulated by 1,25-$(OH)_2D_3$ in growth zone cells and by 24,25-$(OH)_2D_3$ in resting zone cells via a phospholipase A_2-dependent pathway.* While there is a positive correlation in growth zone cells between increased 1,25-$(OH)_2D_3$-dependent PKC specific activity and 1,25-$(OH)_2D_3$-dependent phospholipase A_2 activity, there is a negative correlation in resting zone cells between increased 24,25-$(OH)_2D_3$-dependent PKC specific activity and decreased 24,25-$(OH)_2D_3$-dependent phospholipase A_2 activity. In addition, since the effects of phospholipase A_2 are most likely mediated through arachidonic acid, the product of phospholipase A_2 action, increased levels of arachidonic acid should stimulate PKC specific activity in growth zone cells and inhibit PKC specific activity in resting zone cells. Further downstream products of arachidonic acid metabolism, such as prostaglandin E_2, should have similar cell maturation-dependent and metabolite-specific effects. *To test this hypothesis, cell layer PKC specific activity will be determined after growth zone and resting zone cells are cultured with various phospholipase A_2 activators or inhibitors, arachidonic acid, prostaglandin E_2, and indomethacin, either alone, or in the presence of the target cell-specific vitamin D_3 metabolite.*

MATERIALS AND METHODS

A. Reagents

Both 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ were generous gifts of Dr. Milan Uskokovic of Hoffman-LaRoche (Nutley, NJ). Stock solutions of the vitamin D_3 metabolites were prepared in absolute ethanol and diluted at least 1:5000 (v/v) with culture medium before addition to the cultures. Protein kinase C assay reagents were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent (Smith et al., 1985) obtained from Pierce Chemical Co. (Rockford, IL). Quinacrine, a general inhibitor of phospholipase A_2 (Church et al., 1993), indomethacin, a cyclooxygenase inhibitor, and prostaglandin E_2 were purchased from Sigma Chemical Co. (St. Louis, MO). Arachidonic acid was obtained from Calbiochem (San Diego, CA). The cytosolic selective inhibitor of phospholipase A_2, arachidonyltrifluoromethylketone ($AACOCF_3$; Street et al., 1993), as well as the secretory selective inhibitor of phospholipase A_2, oleyloxyethylphosphorylcholine (OEPC; Magolda and Galbraith, 1989), were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Two phospholipase A_2 activators, melittin (Habermann, 1972) and mastoparan (Argiolas and Pisano, 1983), were also obtained from BIOMOL Research Laboratories.

B. Chondrocyte Cultures

The cell culture model used has been previously described in detail (Boyan et al., 1988). Ribcages were removed from 125g male Sprague-Dawley rats and placed in Dulbecco's Modified Eagle's Medium (DMEM). Using sharp dissection, the resting zone cartilage was separated from the adjacent growth zone cartilage and the intervening tissue discarded. The cartilage was then

sliced and incubated overnight in DMEM containing antibiotics at 37°C with 5% CO_2 in air and 100% humidity. After two 20-minute washes in Hank's Balanced Salt Solution (HBSS), the cells were released from the extracellular matrix by sequential digestions using 1% trypsin for 1 hour and 0.02% collagenase for 3 hours. All enzymes were prepared in HBSS. After complete digestion, the cells were separated from tissue debris by filtration through 40 mesh nylon and collected by centrifugation at 500 x *g* for 10 minutes, resuspended in DMEM, and plated at a density of 10,000 cells/cm^2 for resting zone chondrocytes and 25,000 cells/cm^2 for growth zone chondrocytes. Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-fungizone, and 50μg/ml ascorbic acid (vitamin C) in an atmosphere of 5% CO_2 and 100% humidity at 37°C. Media were changed at 24 and 72 hours and then at 72-hour intervals. At confluence (7-10 days), cells were subcultured using the same plating densities and techniques as those described above and allowed to return to confluence. Confluence was determined visually using an inverted microscope. Third passage, confluent cultures were plated in 24-well tissue culture plates at the same seeding densities and grown to confluence. Previous studies have demonstrated a retention of differential phenotypic markers, both morphologically and biochemically, at this passage (Boyan et al., 1988; 1992).

C. Overall Experimental Approach

Confluent cultures of fourth passage resting zone or growth zone chondrocytes were used in all experiments. Chondrocytes were cultured in 24-well plates and treated for various periods of time with 0.5 ml of control media or media containing 10^{-8} M 1,25-$(OH)_2D_3$ (growth zone cells) or 10^{-8} M 24,25-$(OH)_2D_3$ (resting zone cells) in the absence or presence of various concentrations of phospholipase A_2 activators (melittin or mastoparan), phospholipase A_2 inhibitors (quinacrine, OEPC, or $AACOCF_3$), arachidonic acid, prostaglandin E_2, or indomethacin.

The concentration of the vitamin D_3 metabolites used in this study was selected because prior studies had shown that it induced maximal release of arachidonic acid by the cultures (Schwartz and Boyan, 1988a). Time points were selected based on previous studies (Sylvia et al., 1993) showing that 1,25-$(OH)_2D_3$ exerts its maximal effect on PKC specific activity in growth zone cells at 9 minutes, and is sustained through 90 minutes. In contrast, 24,25-$(OH)_2D_3$ has its maximal effect on PKC specific activity in resting zone cells at 90 minutes, and extends to 270 minutes. After the appropriate incubation period, all conditioned media were removed and the cells washed with phosphate buffered saline (PBS). The cell layers were then 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. The cell layer lysates were assayed for protein content and PKC specific activity as described below.

D. Protein Content

The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent (Smith et al., 1985). This assay can detect protein concentrations in dilute solutions from 0.05 μg/μl to 1.2 μg/μl. The water soluble salt of BCA is a sensitive reagent for copper. The BCA protein assay reagent binds with protein which then reacts with Cu^{2+}, producing a purple colorimetric reaction. In 96-well plates, 100 μl of each sample was added in duplicate, to which an additional 100 μl of BCA reagent was added. After incubating for 30 minutes at 37°C, the absorbance of each well was determined using an EIA Reader (BioRad Inc., Richmond, CA) set at 570 nm and compared to standardized readings obtained with bovine serum albumin.

E. Protein Kinase C Assay

Chondrocyte culture 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. The use of this lipid preparation provides the cofactors and conditions necessary for optimal activity (Bell et al., 1986). To this mixture, a high affinity myelin basic protein peptide and [^{32}P]-ATP (25μCi/ml) were added to a final assay volume of 50μl. Following a 10-minute incubation in a 30°C waterbath, 25 μl from each of the 6 microcentrifuge tubes per sample were spotted onto phosphocellulose discs. The discs were then washed twice for 5 minutes each with 1% phosphoric acid and once with distilled water for 1 minute to remove unincorporated label. The discs were then air dried for 15 minutes prior to placement in scintillation vials containing Ready-Gel cocktail. The amount of radioactive label on the phosphocellulose discs was counted for 1 minute in a Beckman LS6000IC scintillation counter.

F. Effect of Phospholipase A_2 Inhibitors

Previous studies have demonstrated that the phospholipase A_2 inhibitors, quinacrine and OEPC, increase PKC specific activity in resting zone cells (Helm et al., 1996; Boyan et al., 1997b) and that this increase is synergistic when combined with 24,25-$(OH)_2D_3$. In growth zone cells, however, preliminary studies have shown that arachidonyltrifluoromethylketone ($AACOCF_3$), a cytosolic selective inhibitor of phospholipase A_2, inhibits both basal- and 1,25-$(OH)_2D_3$-induced PKC specific activity (Boyan et al., 1997b). To further define the effect of inhibiting phospholipase A_2 on PKC specific activity in control and vitamin D_3-treated cultures in a general, secretory selective, or cytosolic selective manner, quinacrine (Church et al., 1993), OEPC (Magolda and Galbraith, 1989), and $AACOCF_3$ (Street et al., 1993) were used respectively. Growth zone cell cultures were incubated for 9 minutes with DMEM + 10% FBS containing 10^{-8}

M 1,25-$(OH)_2D_3$ ± 0, 0.1, 1.0, or 10 μM quinacrine, OEPC, or $AACOCF_3$ in DMEM + 0.02% PBS. Control cultures were treated with media containing the vitamin D_3 vehicle and the inhibitor vehicle. In addition, the time course of the response was determined by incubating growth zone cells for 9, 90, 270 (4.5 hours), or 1440 (24 hours) minutes with vehicle alone (ethanol + PBS), 10^{-8} M 1,25-$(OH)_2D_3$ + PBS, 10 μM inhibitor + ethanol, or 10^{-8} M 1,25-$(OH)_2D_3$ + 10 μM inhibitor. Resting zone cell cultures were treated for 90 minutes with DMEM + 10% FBS containing 10^{-8} M 24,25-$(OH)_2D_3$ ± 0, 0.1, 1.0, or 10 μM quinacrine, OEPC, or $AACOCF_3$ in DMEM + 0.02% PBS. Control cultures were treated with media containing the vitamin D_3 vehicle and the inhibitor vehicle. In addition, the time course of the response was determined by incubating resting zone cells for 9, 90, 270, or 1440 minutes with vehicle alone (ethanol + PBS), 10^{-8} M 24,25-$(OH)_2D_3$ + PBS, 0.1, 1.0, or 10 μM inhibitor + ethanol, or 10^{-8} M 24,25-$(OH)_2D_3$ + 0.1, 1.0, or 10 μM inhibitor. At the end of the incubation, PKC specific activity in the cell layer was assayed.

G. Effect of Phospholipase A_2 Activators

To evaluate the relationship between the stimulation of phospholipase A_2 and the resulting effect on protein kinase C in control and vitamin D_3-treated cultures, the phospholipase A_2 activators, melittin (Habermann, 1972) and mastoparan (Argiolas and Pisano, 1983) were used. Growth zone cells were treated for 3, 9, 30, or 90 minutes with vehicle alone (ethanol + PBS), 10^{-8} M 1,25-$(OH)_2D_3$, 0.03, 0.3, or 3 μg/ml melittin ± 10^{-8} M 1,25-$(OH)_2D_3$. Resting zone cells cultures were treated for 9, 30, 90, or 270 minutes with vehicle alone (ethanol + PBS), 10^{-8} M 24,25-$(OH)_2D_3$, or 0.03, 0.3, or 3 μg/ml melittin ± 10^{-8} M 24,25-$(OH)_2D_3$. Mastoparan was tested in a similar manner, but at final concentrations of 0.2, 2, or 20 μg/ml. At the end of incubation, PKC specific activity in the cell layer was assayed.

H. Effect of Arachidonic Acid

The effect of exogenous arachidonic acid on PKC specific activity was also examined as a function of dose and time. Growth zone chondrocyte cultures were treated for 9, 90, or 270 minutes with media containing vehicle alone (ethanol + PBS), 10^{-8} M 1,25-$(OH)_2D_3$, 1, 10, or 100 μM arachidonic acid, or a combination of 10^{-8} M 1,25-$(OH)_2D_3$ and arachidonic acid. In resting zone chondrocyte cultures, cells were treated for 9, 90, or 270 minutes with media containing vehicle alone (ethanol + PBS), 10^{-8} M 24,25-$(OH)_2D_3$, 1, 10, or 100 μM arachidonic acid, or a combination of 10^{-8} M 24,25-$(OH)_2D_3$ and arachidonic acid. After incubation, PKC specific activity was measured.

I. Effect of Indomethacin

In order to examine whether the downstream metabolites of arachidonic acid are involved in the regulation of PKC specific activity by 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, indomethacin was added to cultures to prevent prostaglandin E_2 synthesis by blocking the cyclooxygenase pathway. Growth zone chondrocytes were incubated for 9 minutes with 0.01, 0.10, or 1.00 μM indomethacin ± 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-9} M 1,25-$(OH)_2D_3$. Resting zone cells were incubated for 90 minutes with 0.01, 0.10, or 1.00 μM indomethacin ± 10^{-8} M 24,25-$(OH)_2D_3$ or 10^{-9} M 24,25-$(OH)_2D_3$. Protein kinase C specific activity was then measured.

J. Effect on Prostaglandin E_2

To test the specific effects of exogenous prostaglandin E_2 on PKC specific activity, cultures were examined as a function of dose and time. Prostaglandin E_2 was stored as a stock solution in 100% ethanol at -70°C and diluted immediately before use in culture medium. Both growth zone and resting zone chondrocytes were treated with 0.015, 0.060, or 0.23 ng/ml

prostaglandin E_2 for 9 and 90 minutes. At harvest, the cell layers were assayed for PKC specific activity.

K. Statistical Analysis

PKC specific activity data are expressed as the mean pMol phosphate transferred/μg protein/minute ± standard error of the mean (SEM) of six cultures. Observations were validated by a minimum of two independent experimental replications. Differences between groups were determined by ANOVA and significance determined by Bonferroni's modification of the Student's *t*-test using $P < 0.05$ confidence limits.

RESULTS

A. Effect of Phospholipase A_2 Inhibitors

In growth zone chondrocytes, the inhibition of phospholipase A_2 with either quinacrine, OEPC, or $AACOCF_3$ produced a corresponding dose-dependent reduction in PKC specific activity (Figure 1 and Table 1). After 9 minutes of treatment, 0.1 μM quinacrine had no effect on the level of PKC specific activity in either control or 1,25-$(OH)_2D_3$-treated cultures. However, when either 1 or 10 μM quinacrine was added to the cultures, both control and 1,25-$(OH)_2D_3$-treated cultures contained significantly less PKC specific activity. For 1 and 10 μM quinacrine, the reduction in the 1,25-$(OH)_2D_3$-induced PKC specific activity was 32% and 66%, respectively (Figure 1A). The addition of the secretory phospholipase A_2 inhibitor, OEPC, had similar effects. While the addition of 0.1 μM OEPC had no effect on either the control or 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone chondrocytes, the addition of either 1 or 10 μM OEPC reduced the effect of 1,25-$(OH)_2D_3$ by 48% and 56%, respectively (Figure 1B). The effects on PKC specific activity following the addition of the cytosolic phospholipase A_2 inhibitor, $AACOCF_3$, to growth zone cell cultures were similar to those observed for quinacrine and OEPC. Again, the addition of the smallest dose tested, 0.1 μM $AACOCF_3$, had minimal effect on basal PKC specific activity, however, 1,25-$(OH)_2D_3$-stimulated PKC specific activity was significantly reduced by 74% (Table 1). Likewise, both 1 and 10 μM $AACOCF_3$ dose-dependently reduced the effect of 1,25-$(OH)_2D_3$ by 77% and 79%, respectively (Table 1).

Figure 1: Effect of phospholipase A_2 inhibitors on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cells were treated with 0, 0.1, 1 or 10 μM quinacrine (Quin), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9 minutes (upper panel); alternatively, cells were treated with 0, 0.1, 1 or 10 μM OEPC, 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 1,25-$(OH)_2D_3$ alone.

Effect of PA_2 Inhibitors on PKC Activity in GC

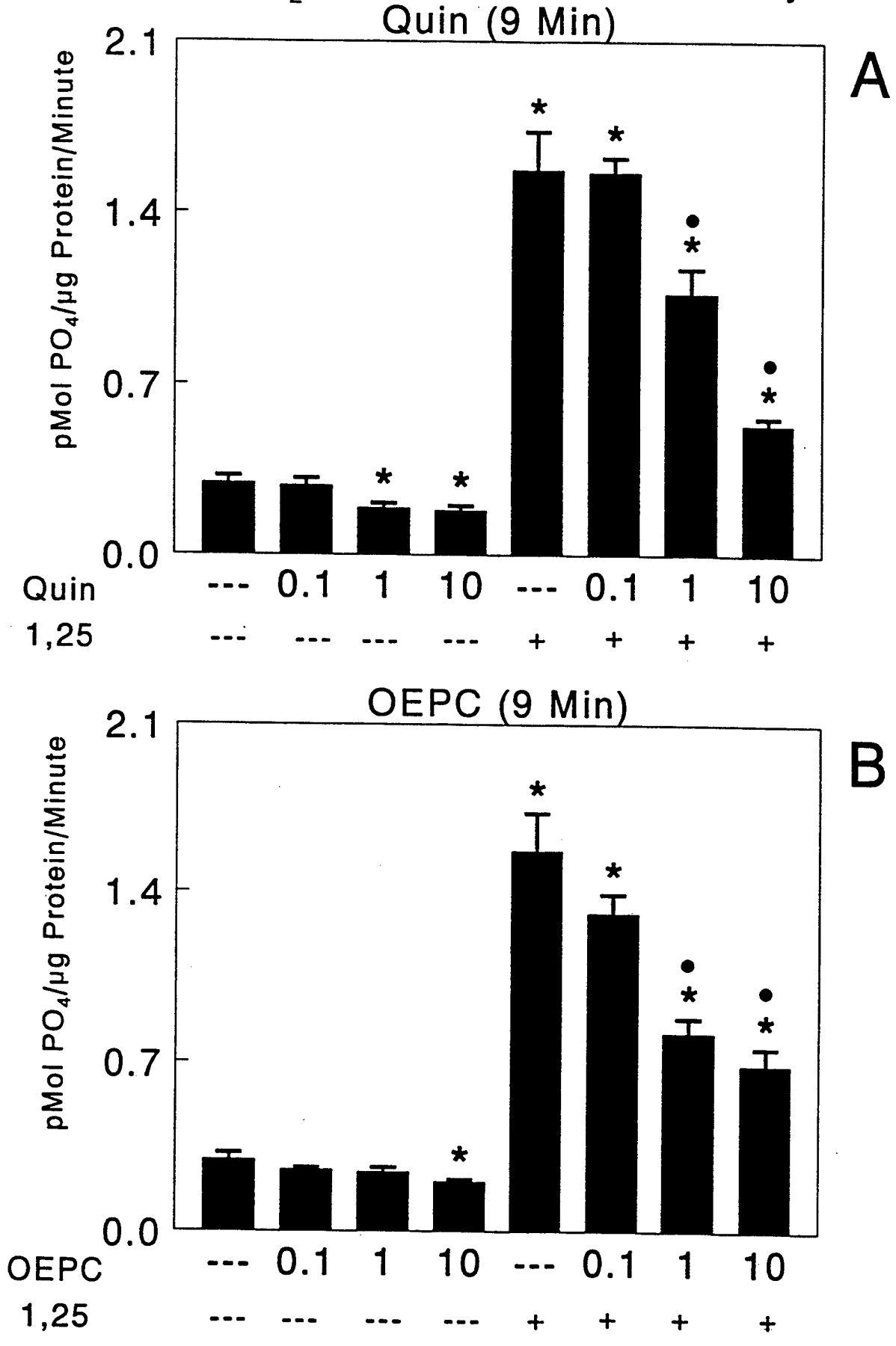


Table 1: Effect of the cytoplasmic phospholipase A_2 inhibitor, $AACOCF_3$, on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cells were treated with 0, 0.1, 1 or 10 μM $AACOCF_3$, 10^{-8} M $1,25\text{-}(OH)_2D_3$, or a combination of the two for 9, 90, 270 or 1440 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; •P<0.05, vs. treatment with $1,25\text{-}(OH)_2D_3$ alone.

Protein Kinase C Specific Activity

(pMol PO_4/μg Protein/Min)

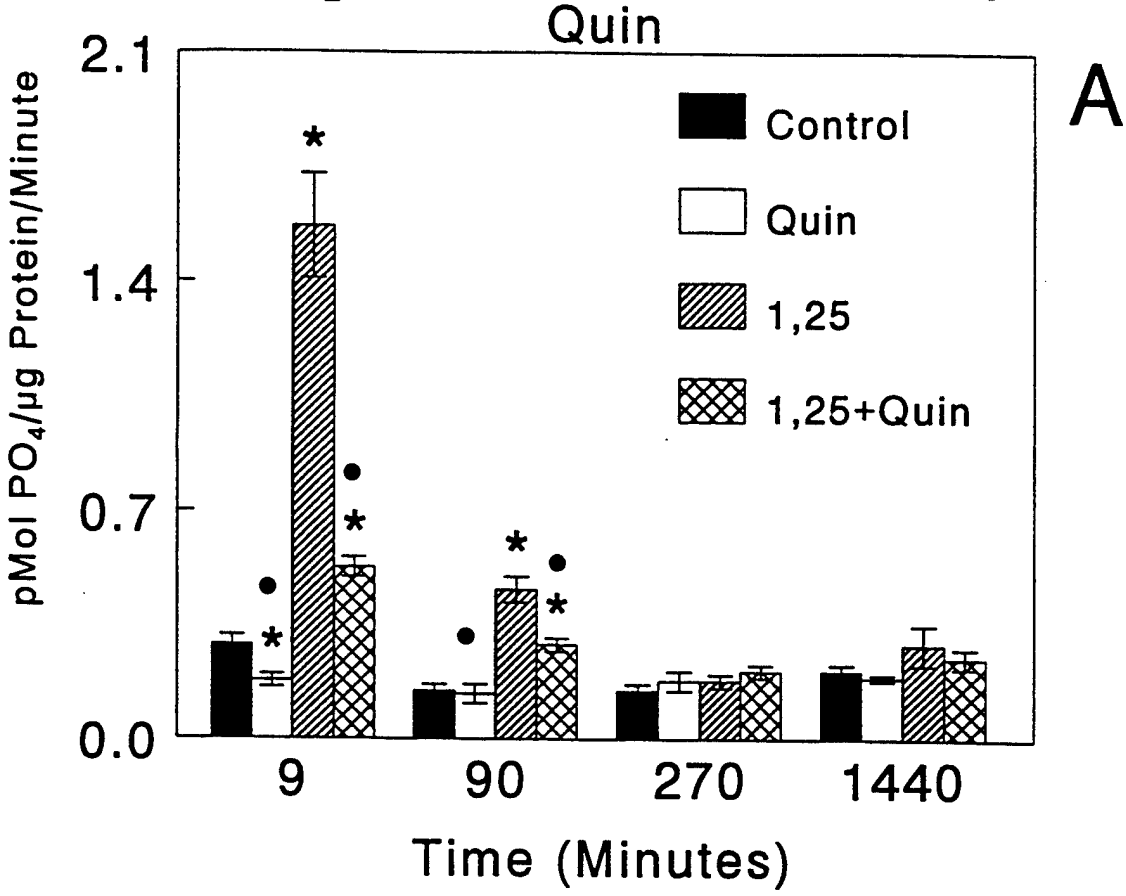
Treatment	9 Min	90 Min	270 Min	1440 Min
Control	0.29 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.28 ± 0.02
+ 0.1 μM $AACOCF_3$	0.40 ± 0.02*	0.16 ± 0.01	0.24 ± 0.03*	0.26 ± 0.03
+ 1 μM $AACOCF_3$	0.23 ± 0.02*	0.16 ± 0.01	0.27 ± 0.03*	0.29 ± 0.03
+ 10 μM $AACOCF_3$	0.24 ± 0.02*	0.17 ± 0.02	0.30 ± 0.02*	0.25 ± 0.01*
10^{-8} M $1,25\text{-}(OH)_2D_3$	1.57 ± 0.16*	0.46 ± 0.04*	0.18 ± 0.02*	0.41 ± 0.06*
+ 0.1 μM $AACOCF_3$	0.41 ± 0.02••	0.15 ± 0.01•	0.24 ± 0.23••	0.14 ± 0.01••
+ 1 μM $AACOCF_3$	0.35 ± 0.05••	0.12 ± 0.01••	0.23 ± 0.02••	0.29 ± 0.05•
+ 10 μM $AACOCF_3$	0.33 ± 0.05••	0.10 ± 0.01••	0.10 ± 0.01••	0.28 ± 0.02•

In growth zone chondrocyte cultures, the effect of the phospholipase A_2 inhibitors on PKC specific activity was also time-dependent (Figure 2 and Table 1). While the addition of 10 μM quinacrine decreased the basal level of PKC specific activity after 9 minutes of treatment, the same dose had no effect after 90, 270, or 1440 minutes. At 9 and 90 minutes, 10 μM quinacrine significantly reduced the 1,25-$(OH)_2D_3$-induced stimulation of PKC specific activity by 66% and 37%, respectively (Figure 2A). The same dose had no effect on the 1,25-$(OH)_2D_3$-dependent PKC specific activity at 270 or 1440 minutes. In a similar manner, 10 μM OEPC or 10 μM $AACOCF_3$ also significantly reduced basal PKC specific activity at 9 minutes, but had no effect at 90, 270, or 1440 minutes. At 9 and 90 minutes, the 1,25-$(OH)_2D_3$-induced stimulation of PKC specific activity was reduced by 56% and 59%, respectively, following the addition of 10 μM OEPC (Figure 2B), and 79% and 78%, respectively, following the addition of 10 μM $AACOCF_3$ (Table 1).

In contrast to the dose-dependent inhibitory effect on PKC specific activity in growth zone chondrocyte cultures, the addition of the same phospholipase A_2 inhibitors had a dose-dependent stimulatory effect when added to resting zone chondrocyte cultures (Figure 3 and Table 2). After 90 minutes of treatment, 0.1 μM quinacrine had no effect on PKC specific activity in either the control or 24,25-$(OH)_2D_3$-treated cultures. However, both 1 and 10 μM quinacrine significantly increased basal PKC specific activity and enhanced the effect of 24,25-$(OH)_2D_3$ by 33% and 117%, respectively (Figure 3A). Likewise, 0.1 μM OEPC had no effect on PKC specific activity in control or 24,25-$(OH)_2D_3$-treated cultures, but 1 and 10 μM OEPC enhanced the 24,25-$(OH)_2D_3$-dependent stimulation of PKC by 39% and 93%, respectively (Figure 3B). Again at 90

Figure 2: Effect of phospholipase A_2 inhibitors on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes over time. Confluent, fourth passage growth zone cells were treated with 10 μM quinacrine (Quin), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9, 90, 270 or 1440 minutes (upper panel); alternatively, cells were treated with 10 μM OEPC, 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9, 90, 270 or 1440 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 1,25-$(OH)_2D_3$ alone.

Effect of PA_2 Inhibitors on PKC Activity in GC



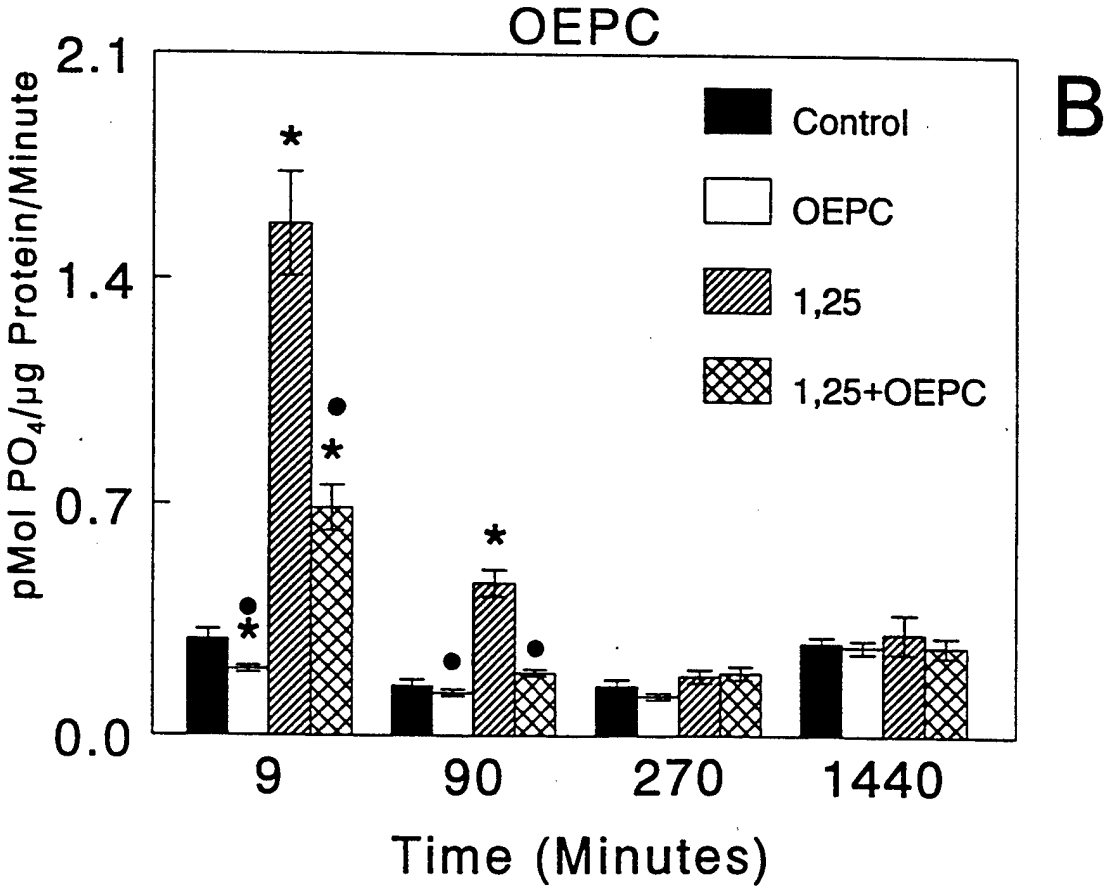


Figure 3: Effect of phospholipase A_2 inhibitors on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cells were treated with 0, 0.1, 1 or 10 μM quinacrine (Quin), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 90 minutes (upper panel); alternatively, cells were treated with 0, 0.1, 1 or 10 μM OEPC, 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 90 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 24,25-$(OH)_2D_3$ alone.

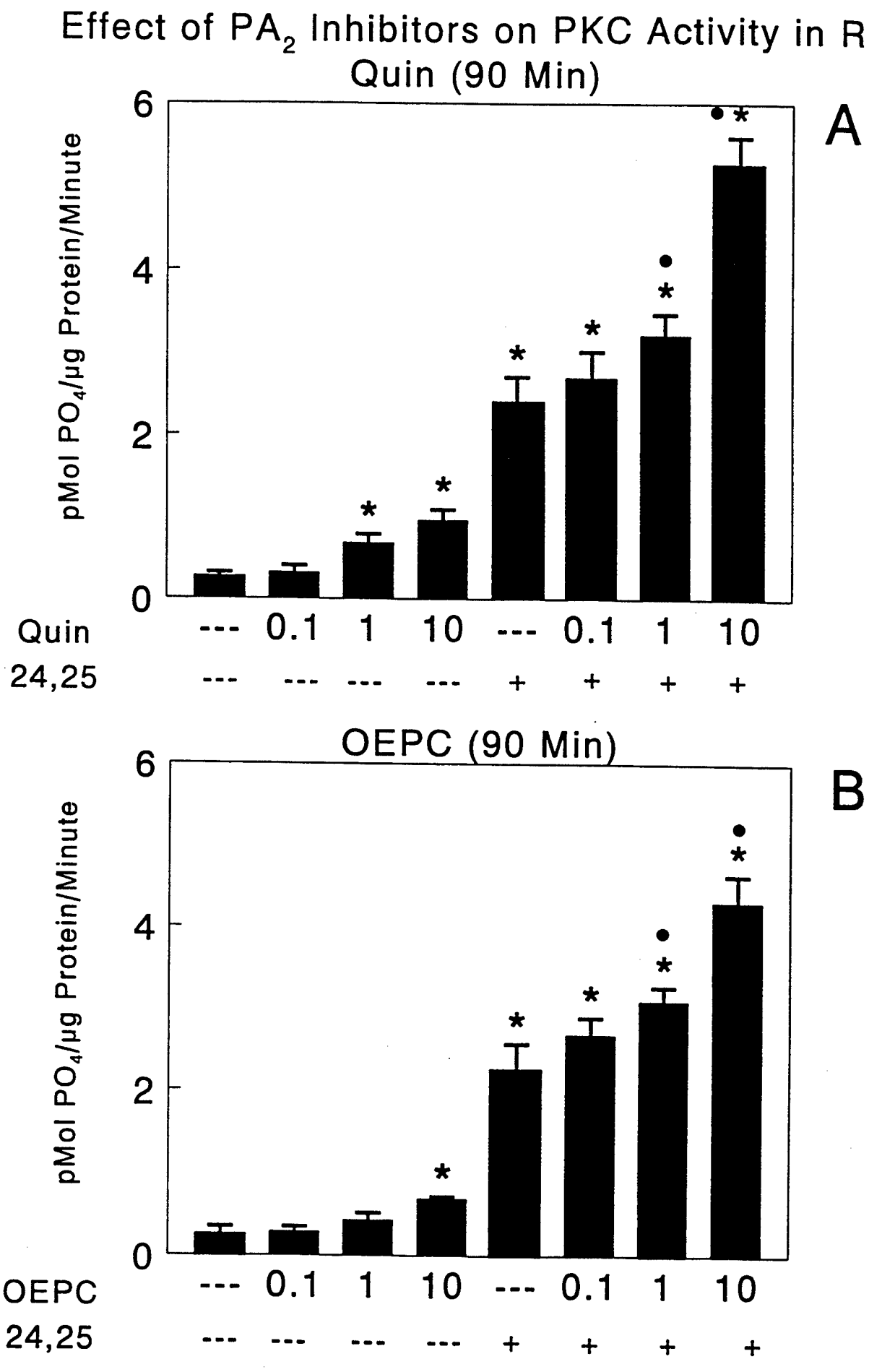

Effect of PA2 Inhibitors on PKC Activity in RC
Quin (90 Min)
A
pMol PO4/µg Protein/Minute
6
4
2
0
Quin
24,25
--- 0.1 1 10 --- 0.1 1 10
--- --- --- --- + + + +
OEPC (90 Min)
B
pMol PO4/µg Protein/Minute
6
4
2
0
OEPC
24,25
--- 0.1 1 10 --- 0.1 1 10
--- --- --- --- + + + +

Table 2: Effect of the cytoplasmic phospholipase A_2 inhibitor, $AACOCF_3$, on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cells were treated with 0, 0.1, 1 or 10 μM $AACOCF_3$, 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 9, 90 or 270 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; *P<0.05, vs. treatment with 24,25-$(OH)_2D_3$ alone.

Protein Kinase C Specific Activity
(pMol PO_4/μg Protein/Min)

Treatment	9 Min	90 Min	270 Min
Control	3.99 ± 0.61	3.46 ± 0.67	6.02 ± 2.63
+ 0.1 μM $AACOCF_3$	10.61 ± 1.10*	14.69 ± 1.30*	10.87 ± 1.40*
+ 1 μM $AACOCF_3$	17.38 ± 0.68*	19.88 ± 1.07*	10.40 ± 0.81*
+ 10 μM $AACOCF_3$	4.78 ± 0.57*	32.32 ± 3.07*	10.64 ± 0.70*
10^{-8} M 24,25-$(OH)_2D_3$	4.77 ± 0.33*	23.04 ± 2.16*	10.55 ± 2.20*
+ 0.1 μM $AACOCF_3$	5.93 ± 1.09**	16.42 ± 0.92**	10.82 ± 3.02*
+ 1 μM $AACOCF_3$	5.43 ± 0.38**	31.75 ± 1.74**	11.60 ± 0.53*
+ 10 μM $AACOCF_3$	7.14 ± 0.51**	40.69 ± 2.91**	13.04 ± 0.85**

minutes, the addition of $AACOCF_3$ to resting zone chondrocyte cultures dose-dependently stimulated the basal level of PKC specific activity by 324%, 475%, and 834%, respectively, for 0.1, 1, and 10 μM $AACOCF_3$ (Table 2). While 0.1 μM $AACOCF_3$ had no effect on the level of PKC specific activity in 24,25-$(OH)_2D_3$-treated cultures, 1 and 10 μM $AACOCF_3$ stimulated this activity by 38% and 77%, respectively (Table 2).

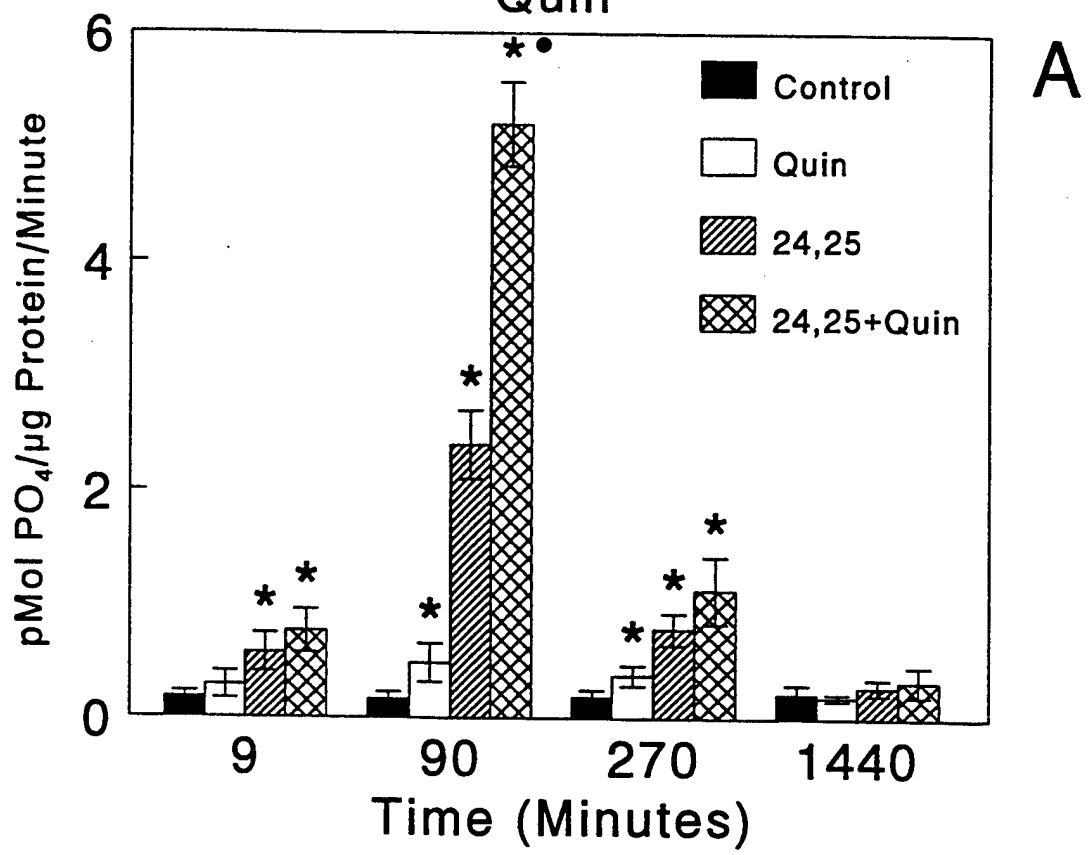
Further, the effect of the phospholipase A_2 inhibitors on PKC specific activity in resting zone chondrocyte cultures was time-dependent (Figure 4). The addition of 10 μM quinacrine significantly increased the basal level of PKC specific activity after 90 or 270 minutes, but had no effect after 9 or 1440 minutes. At 90 minutes, 10 μM quinacrine significantly enhanced the 24,25-$(OH)_2D_3$-induced stimulation of PKC specific activity by 117% (Figure 4A). In a similar manner, 10 μM OEPC or 10 μM $AACOCF_3$ also stimulated basal PKC specific activity at 90 minutes, but had minimal effect at 9, 270, or 1440 minutes. At 90 minutes, the 24,25-$(OH)_2D_3$-induced PKC specific activity was enhanced by 93% and 77%, respectively, following the addition of either 10 μM OEPC (Figure 4B) or 10 μM $AACOCF_3$ (Table 2).

B. Effect of Phospholipase A_2 Activators

When growth zone chondrocyte cultures were treated with the phospholipase A_2 activators, melittin and mastoparan, there was a dose-dependent increase in PKC specific activity (Figure 5). After 9 minutes of treatment, there was a slight, but statistically significant increase in the basal level of PKC specific activity in cultures treated with 0.3 or 3 μg/ml melittin (Figure 5A). When growth zone cells were treated with either 0.3 or 3 μg/ml melittin plus 1,25-$(OH)_2D_3$ for 9 minutes, PKC specific activity was significantly increased compared to cultures treated with

Figure 4: Effect of phospholipase A_2 inhibitors on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes over time. Confluent, fourth passage resting zone cells were treated with 10 μM quinacrine (Quin), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 9, 90, 270 or 1440 minutes (upper panel); alternatively, cells were treated with 10 μM OEPC, 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 9, 90, 270 or 1440 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 24,25-$(OH)_2D_3$ alone.

Effect of PA_2 Inhibitors on PKC Activity in RC



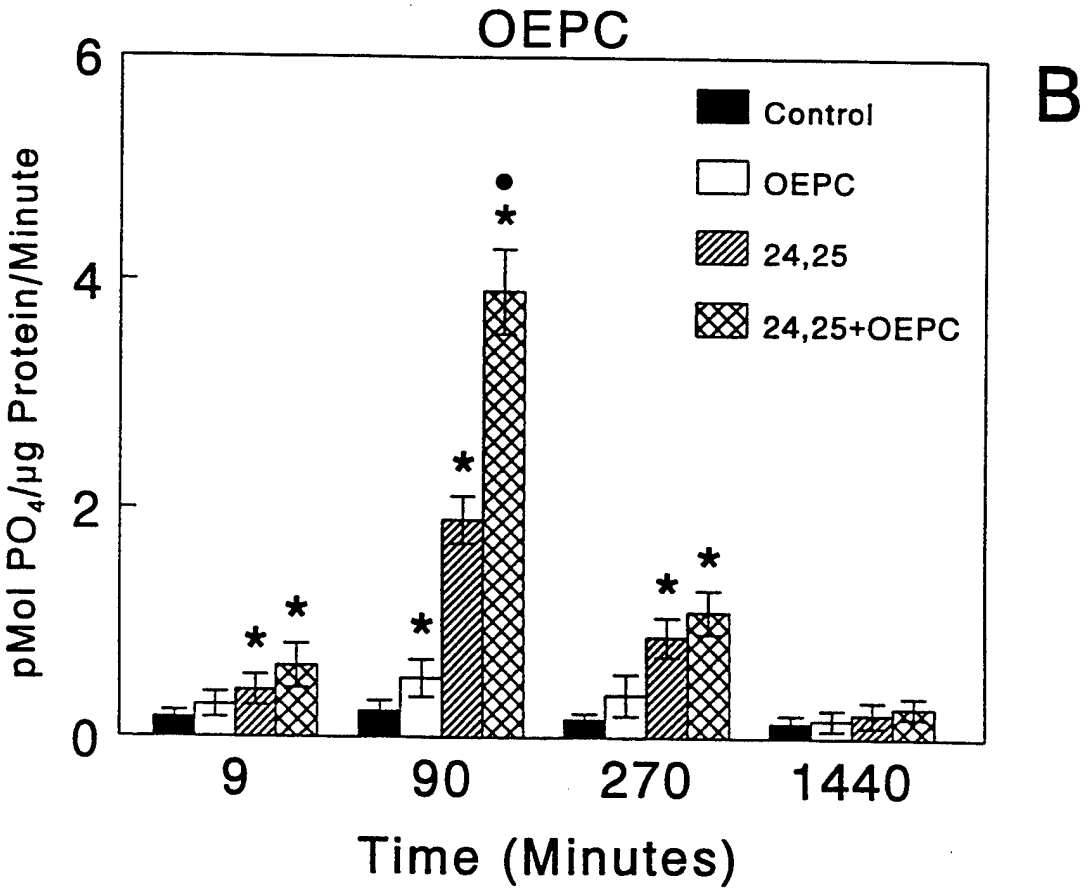
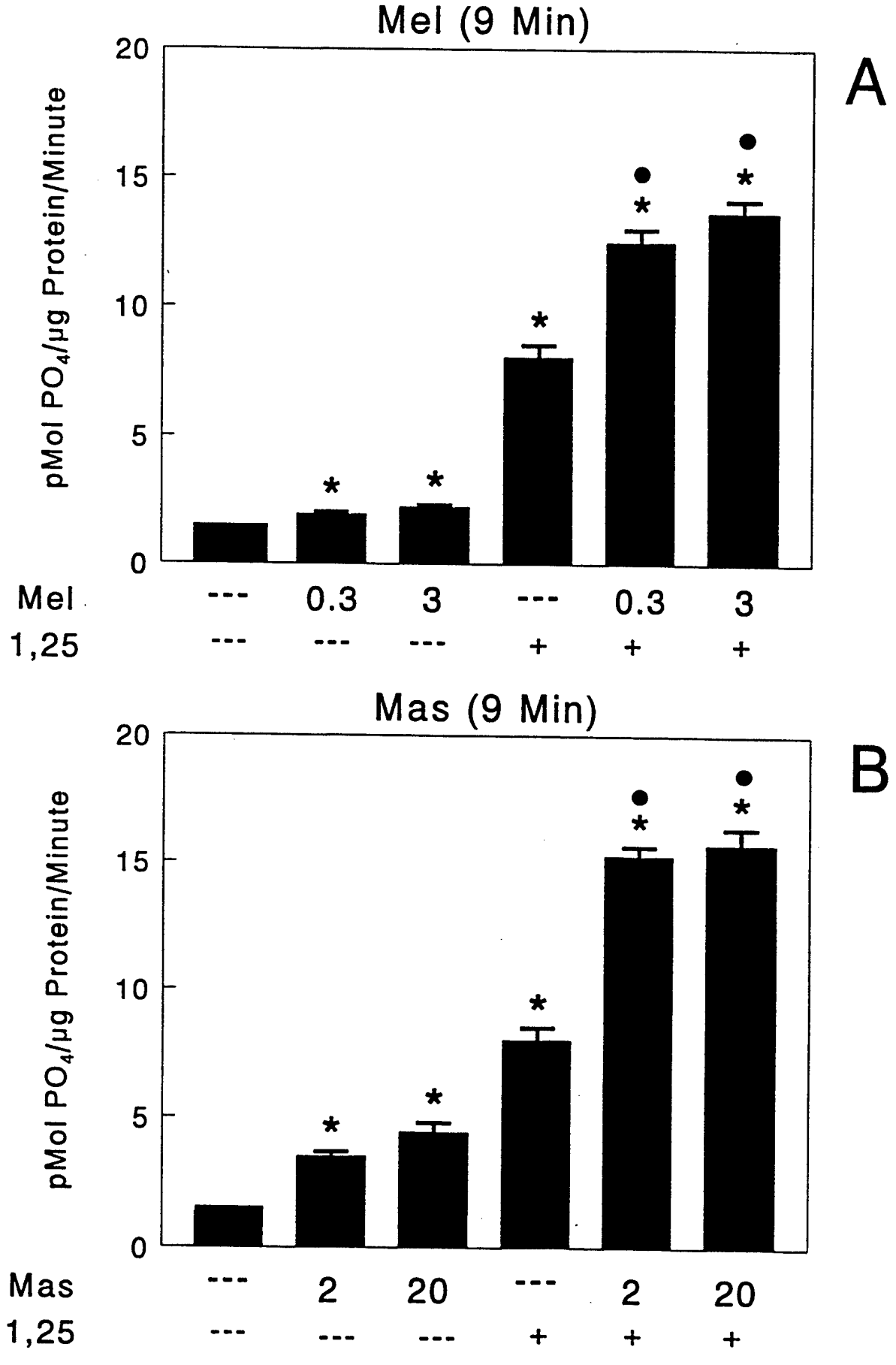


Figure 5: Effect of phospholipase A_2 activators on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cells were treated with 0, 0.3 or 3 μg/ml melittin (Mel), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9 minutes (upper panel); alternatively, cells were treated with 0, 2 or 20 μg/ml mastoparan (Mas), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 9 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; •$P<0.05$, vs. treatment with 1,25-$(OH)_2D_3$ alone.

Effect of PA_2 Activators on PKC Activity in GC



1,25-$(OH)_2D_3$ alone (Figure 5A). PKC specific activity was increased 55% and 70% in 1,25-$(OH)_2D_3$-treated cultures exposed to 0.3 or 3 μg/ml melittin, respectively.

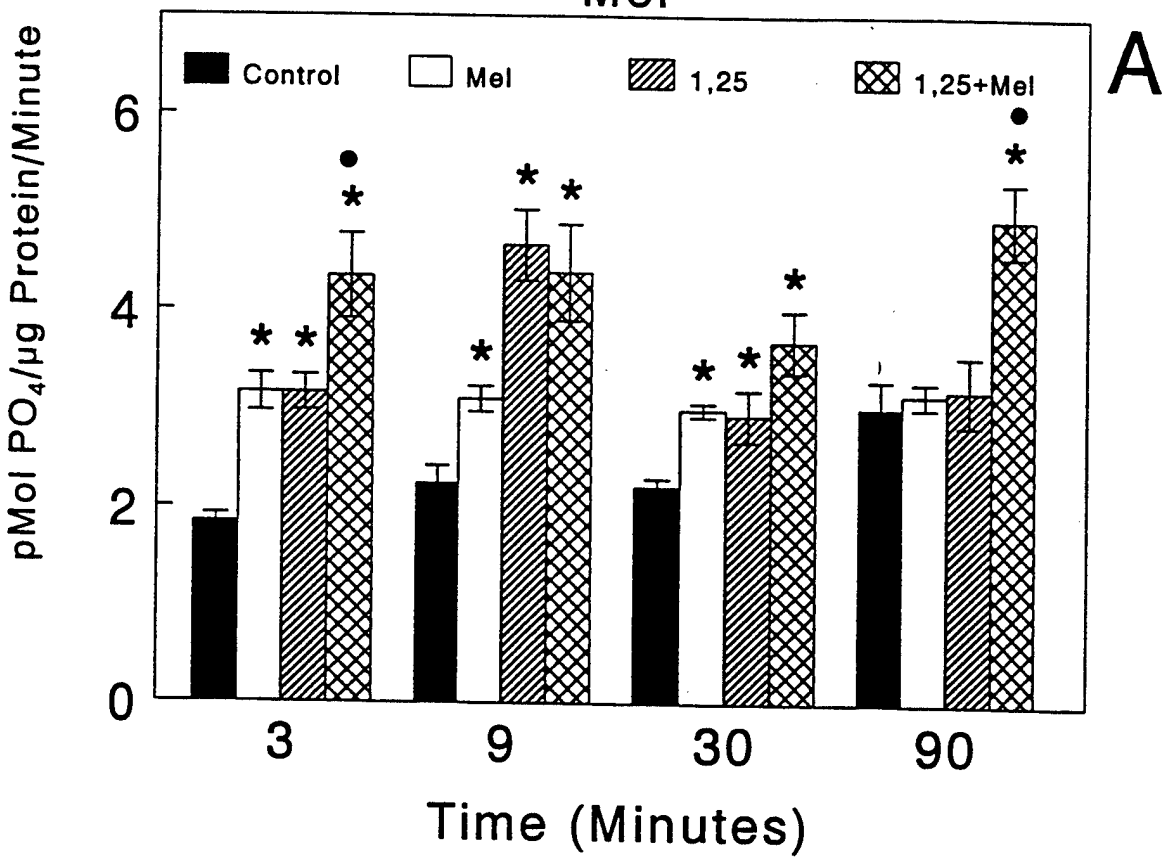
Mastoparan also stimulated PKC specific activity in both control and 1,25-$(OH)_2D_3$-treated growth zone chondrocyte cultures (Figure 5B). There was a statistically significant increase in PKC specific activity of 161% and 230% in control cultures treated with 2 and 20 μg/ml mastoparan, respectively. In a fashion similar to that seen with melittin, mastoparan significantly increased the 1,25-$(OH)_2D_3$-induced PKC specific activity over that seen for cultures treated with 1,25-$(OH)_2D_3$ alone. PKC specific activity was increased 90% and 95% in 1,25-$(OH)_2D_3$-treated cultures exposed to 2 and 20 μg/ml mastoparan, respectively.

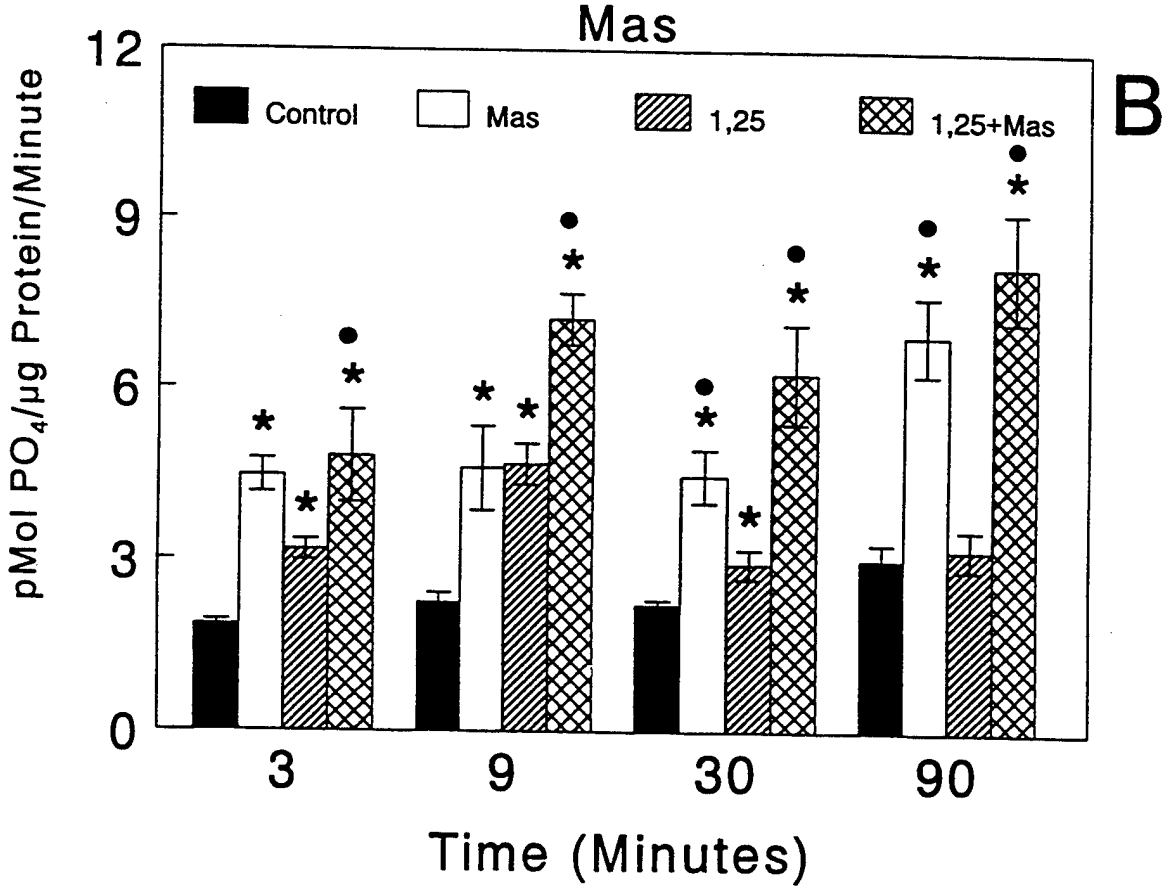
The effects of melittin and mastoparan in growth zone chondrocyte cultures were also time-dependent (Figure 6). When growth zone cell cultures were exposed to 3 μg/ml melittin, the basal level of PKC specific activity was significantly increased by 3 minutes, and this increase remained stable through 30 minutes (Figure 6A). 1,25-$(OH)_2D_3$ alone also increased PKC specific activity at 3, 9, and 30 minutes of treatment, with maximal stimulation occurring at 9 minutes. Further, when growth zone cells were treated with both 3 μg/ml melittin and 1,25-$(OH)_2D_3$, there was an additional increase in PKC specific activity after 3, 30, and 90 minutes that was significantly higher than that seen with 1,25-$(OH)_2D_3$ alone with the same length of treatment (Figure 6A). At 9 minutes, however, comparable increases in PKC specific activity were observed following treatment with 1,25-$(OH)_2D_3$ alone or 3 μg/ml melittin and 1,25-$(OH)_2D_3$ (Figure 6A).

Mastoparan also increased PKC specific activity in growth zone chondrocytes, but the time course of the effect was different than seen with melittin (Figure 6B). The addition of 20 μg/ml mastoparan stimulated PKC specific activity by 3 minutes and remained stable through 30

Figure 6: Effect of phospholipase A_2 activators on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes over time. Confluent, fourth passage growth zone cells were treated with 3 μg/ml melittin (Mel), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 3, 9, 30 or 90 minutes (upper panel); alternatively, cells were treated with 20 μg/ml mastoparan (Mas), 10^{-8} M 1,25-$(OH)_2D_3$, or a combination of the two for 3, 9, 30 or 90 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 1,25-$(OH)_2D_3$ alone.

Effect of PA_2 Activators on PKC Activity in GC



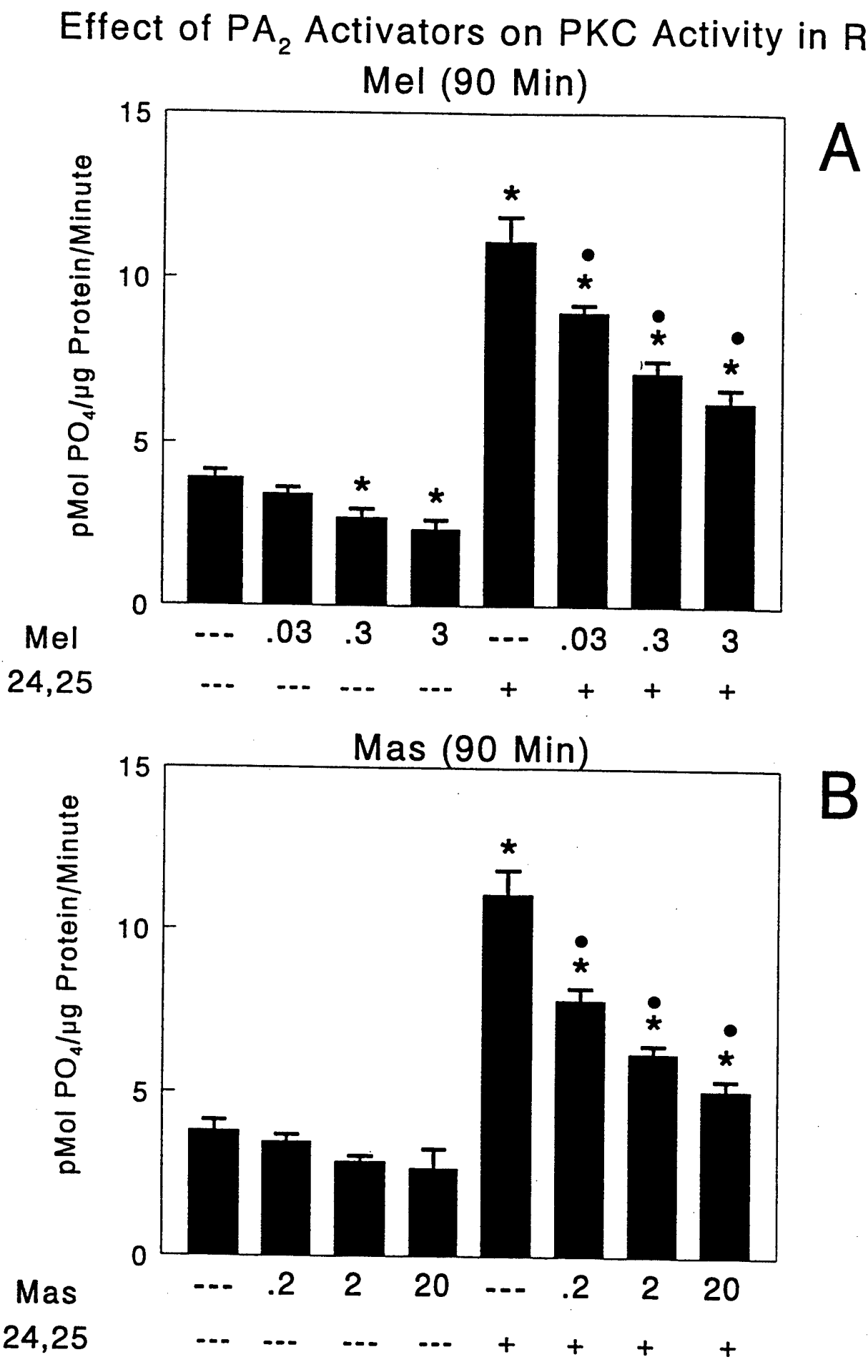


minutes, however, a further increase occurred between 30 and 90 minutes (Figure 6B). 1,25-$(OH)_2D_3$ also caused a time-dependent increase in PKC specific activity at 3, 9 and 30 minutes with maximal stimulation at 9 minutes (Figure 6B). When the cells were treated with both 20 μg/ml mastoparan and 1,25-$(OH)_2D_3$, the effect on PKC specific activity was significantly greater than that seen with either agent alone after 9 minutes and greater than 1,25-$(OH)_2D_3$ alone at 3, 30 and 90 minutes (Figure 6B).

In resting zone chondrocyte cultures, the phospholipase A_2 activators, melittin and mastoparan, caused a dose-dependent decrease in PKC specific activity (Figure 7). After 90 minutes of treatment, there was a slight, but statistically significant decrease in the basal level of PKC specific activity in cultures treated with 0.3 or 3 μg/ml melittin (Figure 7A). When resting zone cells were treated with either 0.03, 0.3, or 3 μg/ml melittin plus 24,25-$(OH)_2D_3$ for 90 minutes, there was a significant dose-dependent decrease in the 24,25-$(OH)_2D_3$-induced PKC specific activity compared to cultures treated with 24,25-$(OH)_2D_3$ alone (Figure 7A). PKC specific activity was decreased 19%, 36% and 44% in 24,25-$(OH)_2D_3$-treated cultures exposed to 0.03, 0.3 or 3 μg/ml melittin, respectively.

Mastoparan also inhibited both basal and 24,25-$(OH)_2D_3$-induced PKC specific activities in resting zone chondrocyte cultures (Figure 7B). In control cultures, the addition of mastoparan resulted in a slight, but statistically insignificant dose-dependent decrease in PKC specific activity. In a similar fashion to melittin, mastoparan significantly decreased 24,25-$(OH)_2D_3$-induced PKC specific activity over that seen for cultures treated with 24,25-$(OH)_2D_3$ alone. PKC specific activity was decreased 30%, 44% and 54% in 24,25-$(OH)_2D_3$-treated cultures exposed to 0.2, 2 or 20 μg/ml mastoparan, respectively.

Figure 7: Effect of phospholipase A_2 activators on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cells were treated with 0, 0.03, 0.3 or 3 μg/ml melittin (Mel), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 90 minutes (upper panel); alternatively, cells were treated with 0, 0.2, 2 or 20 μg/ml mastoparan (Mas), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 90 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; °$P<0.05$, vs. treatment with 24,25-$(OH)_2D_3$ alone.

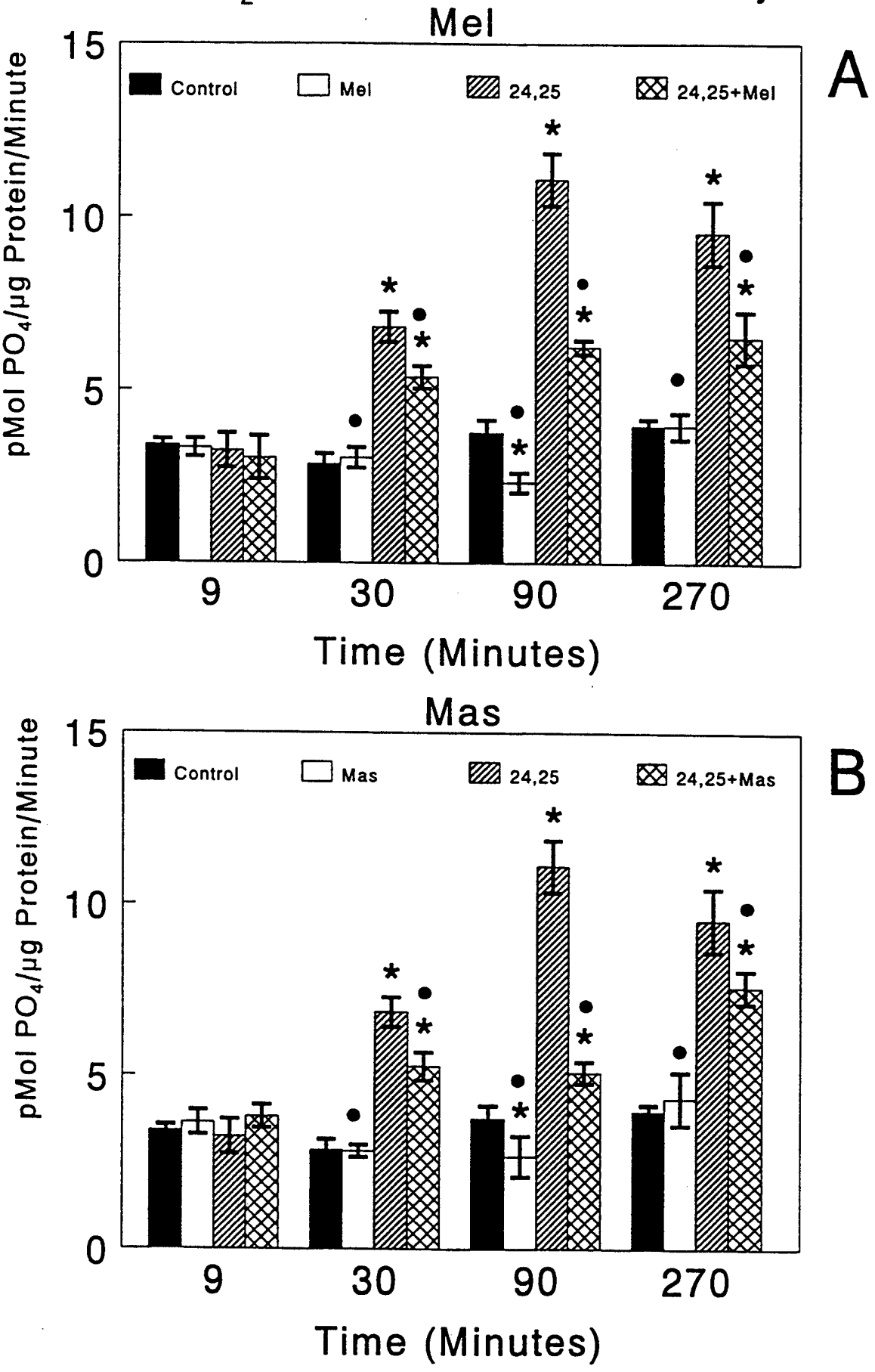

Effect of PA2 Activators on PKC Activity in RC
Mel (90 Min)
A
pMol PO4/µg Protein/Minute
15
10
5
0
Mel
24,25
--- .03 .3 3 --- .03 .3 3
--- --- --- --- + + + +
Mas (90 Min)
B
pMol PO4/µg Protein/Minute
15
10
5
0
Mas
24,25
--- .2 2 20 --- .2 2 20
--- --- --- --- + + + +

In resting zone chondrocyte cultures, the effects of the phospholipase A_2 activators were also time-dependent (Figure 8). When resting zone cell cultures were exposed to 3 μg/ml melittin, the basal level of PKC specific activity was significantly reduced at only the 90 minute time point (Figure 8A). By contrast, the addition of 24,25-$(OH)_2D_3$ alone to resting zone cell cultures stimulated PKC specific activity at 30, 90 and 270 minutes. However, when these cultures were treated with both 3 μg/ml melittin and 24,25-$(OH)_2D_3$, the 24,25-$(OH)_2D_3$-induced PKC specific activity was significantly reduced by 17%, 73% and 55% at 30, 90 and 270 minutes, respectively (Figure 8A). Maximal inhibition of 24,25-$(OH)_2D_3$-stimulated PKC specific activity occurred at 90 minutes.

In a fashion similar to melittin, mastoparan also blocked PKC specific activity in resting zone chondrocytes, again only significant at 90 minutes (Figure 8B). By contrast, 24,25-$(OH)_2D_3$ treated cultures contained increased PKC specific activity at 30, 90 and 270 minutes with maximal stimulation at 90 minutes (Figure 8B). In addition, when the cells were treated with both 20 μg/ml mastoparan and 24,25-$(OH)_2D_3$, the 24,25-$(OH)_2D_3$-induced PKC specific activity was significantly reduced by 42%, 80% and 35% at 30, 90 and 270 minutes, respectively (Figure 8B). Again, maximal inhibition of 24,25-$(OH)_2D_3$-stimulated PKC specific activity occurred at 90 minutes.

Figure 8: Effect of phospholipase A_2 activators on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes over time. Confluent, fourth passage resting zone cells were treated with 3 μg/ml melittin (Mel), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 9, 30, 90 or 270 minutes (upper panel); alternatively, cells were treated with 20 μg/ml mastoparan (Mas), 10^{-8} M 24,25-$(OH)_2D_3$, or a combination of the two for 9, 30, 90 or 270 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; °$P<0.05$, vs. treatment with 24,25-$(OH)_2D_3$ alone.

Effect of PA_2 Activators on PKC Activity in RC



C. Effect of Arachidonic Acid

The addition of arachidonic acid to growth zone chondrocytes for 9 or 270 minutes caused a significant dose-dependent increase in PKC specific activity which was maximal at 100 μM (Figure 9). When 1,25-$(OH)_2D_3$ alone was added to the cultures, a significant increase in PKC specific activity was observed after 9 minutes, but not 270 minutes. While treatment of the cultures with both arachidonic acid and 1,25-$(OH)_2D_3$ for 9 minutes resulted in an additive effect on PKC specific activity (Figure 9A), there was a synergistic increase in PKC specific activity when the combination treatment was extended to 270 minutes (Figure 9B).

The effects of arachidonic acid on growth zone chondrocytes were also time-dependent (Figure 10). After 9 minutes, 100 μM arachidonic acid and 1,25-$(OH)_2D_3$ had comparable effects on PKC specific activity, and in combination, an additive increase was observed. At 90 minutes, the effect of 1,25-$(OH)_2D_3$ is reduced, but the effect of 100 μM arachidonic acid remains the same as seen at 9 minutes. When 100 μM arachidonic acid and 1,25-$(OH)_2D_3$ were combined, there was a synergistic increase in PKC specific activity that was comparable to that observed at 9 minutes. Although 1,25-$(OH)_2D_3$ had no effect on PKC specific activity, and 100 μM arachidonic acid had a reduced effect on PKC specific activity at 270 minutes, together the combination synergistically increased PKC specific activity to levels comparable to 9 minutes.

In resting zone chondrocyte cultures, only the 100 μM arachidonic acid dose had any inhibitory effect on PKC specific activity at both 90 and 270 minutes (Figure 11). When the cultures were treated with 24,25-$(OH)_2D_3$ alone, there was a significant increase in PKC specific activity at both time points, with a four-fold greater increase observed at 90 minutes.

Figure 9: Effect of arachidonic acid (AA) on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cells were treated with 1, 10 or 100 μM AA in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ for 9 minutes (upper panel) or 270 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; °$P<0.05$, vs. treatment with 1,25-$(OH)_2D_3$ alone.

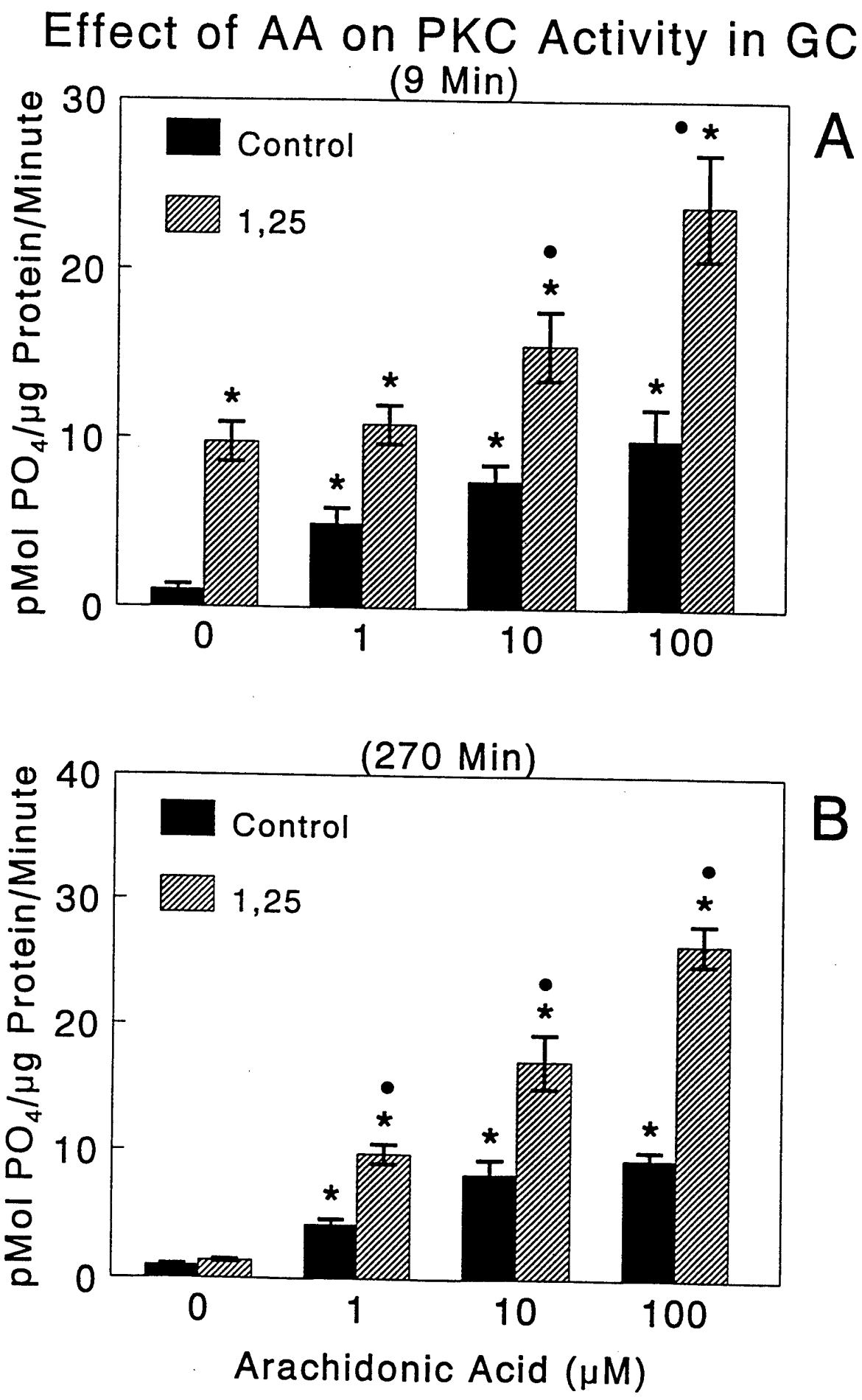

Effect of AA on PKC Activity in GC
(9 Min)
A
Control
1,25
pMol PO4/µg Protein/Minute
30
20
10
0
0
1
10
100
(270 Min)
B
Control
1,25
pMol PO4/µg Protein/Minute
40
30
20
10
0
0
1
10
100
Arachidonic Acid (µM)

Figure 10: Effect of arachidonic acid (AA) on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes over time. Confluent, fourth passage growth zone cells were treated with control media, 10^{-8} M 1,25-$(OH)_2D_3$ alone, 100 μM AA alone, or 10^{-8} M 1,25-$(OH)_2D_3$ plus 100 μM AA together for 9, 90 or 270 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; ˚P<0.05, vs. AA or 1,25-$(OH)_2D_3$ alone.

Effect of Treatment Time with AA on PKC Activity in GC

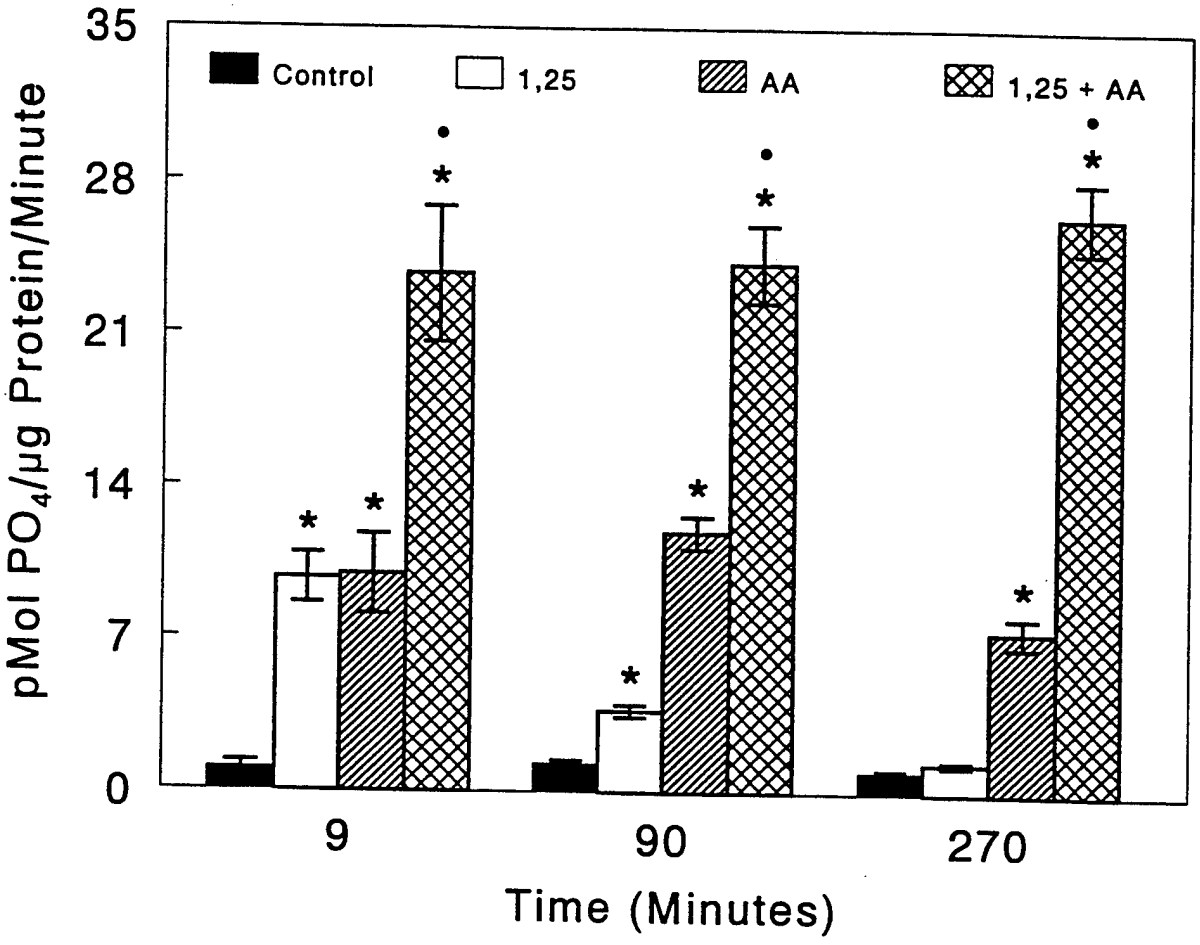
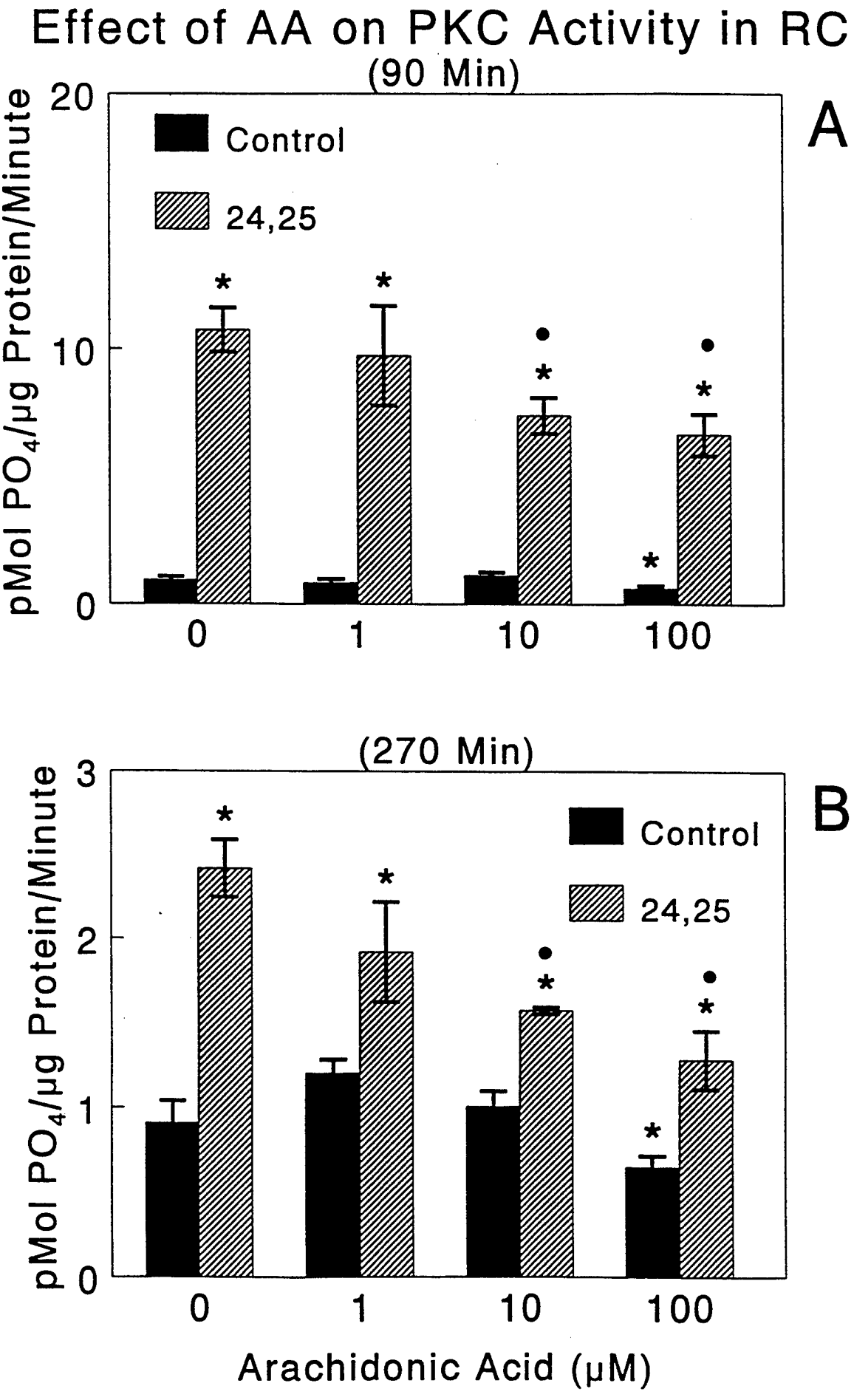


Figure 11: Effect of arachidonic acid (AA) on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cells were treated with 1, 10 or 100 μM AA in the absence or presence of 10^{-8} M 24,25-$(OH)_2D_3$ for 90 minutes (upper panel) or 270 minutes (lower panel). At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control; *$P<0.05$, vs. treatment with 24,25-$(OH)_2D_3$ alone.


Effect of AA on PKC Activity in RC
(90 Min)
A
pMol PO4/µg Protein/Minute
20
10
0
Control
24,25
0
1
10
100
(270 Min)
B
3
2
1
0
Control
24,25
Arachidonic Acid (µM)

The combination of arachidonic acid and 24,25-$(OH)_2D_3$ resulted in a dose-dependent reduction in the 24,25-$(OH)_2D_3$-stimulated PKC specific activity which was significant for 10 and 100 μM arachidonic acid at both 90 and 270 minutes.

The inhibitory effect on PKC specific activity in resting zone cells was not time-dependent (Figure 12). Both basal and 24,25-$(OH)_2D_3$-stimulated PKC specific activity were consistently reduced by approximately 50% following treatment with 100 μM arachidonic acid, thus the greatest effect observed was at 90 minutes when maximal stimulation of PKC by 24,25-$(OH)_2D_3$ has occurred.

D. Effect of Indomethacin

The addition of indomethacin, a cyclooxygenase inhibitor, had no effect on basal PKC specific activity in growth zone chondrocyte cultures (Figure 13). However, at 9 minutes, the combination of indomethacin with 1,25-$(OH)_2D_3$ caused a significant dose-dependent reduction in the level of PKC in the cultures for every concentration of indomethacin and 1,25-$(OH)_2D_3$ tested. In cultures treated with 10^{-9} M 1,25-$(OH)_2D_3$, the addition of 0.1, 1 and 10 μM indomethacin caused an inhibition of PKC specific activity by 32%, 38% and 49%, respectively. In a similar manner, 10^{-8} M 1,25-$(OH)_2D_3$ treated cultures had PKC specific activity reduced by 28%, 41% and 50%, respectively, following the addition of 0.1, 1 and 10 μM indomethacin.

In resting zone cell cultures, the addition of indomethacin caused a dose-dependent increase in PKC specific activity which was significant for 1 and 10 μM concentrations (Figure 14). At 90 minutes, both concentrations of 24,25-$(OH)_2D_3$ caused a significant increase in PKC specific activity. In addition, when indomethacin was combined with 24,25-$(OH)_2D_3$-treated

Figure 12: Effect of arachidonic acid (AA) on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes over time. Confluent, fourth passage resting zone cells were treated with control media, 10^{-8} M 24,25-$(OH)_2D_3$ alone, 100 μM AA alone, or 10^{-8} M 24,25-$(OH)_2D_3$ plus 100 μM AA together for 9, 90 or 270 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; °P<0.05, vs. AA or 24,25-$(OH)_2D_3$ alone.

Effect of Treatment Time with AA on PKC Activity in RC

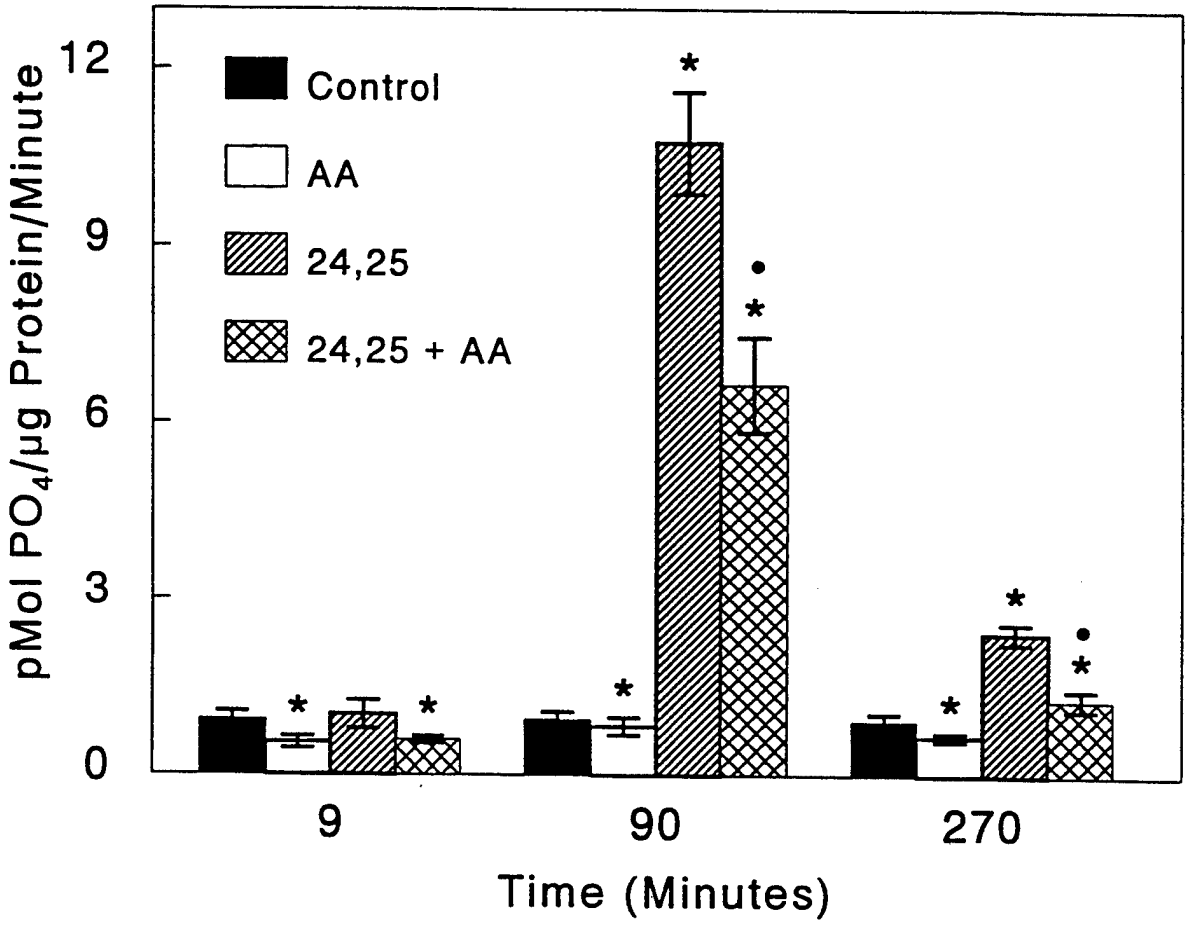


Figure 13: Effect of indomethacin on basal and vitamin D_3 metabolite-stimulated PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cultures were treated with 0, 0.1, 1 or 10 μM indomethacin in the absence or presence of 10^{-9} M or 10^{-8} M 1,25-$(OH)_2D_3$ for 9 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; °P<0.05, vs. cultures without indomethacin.

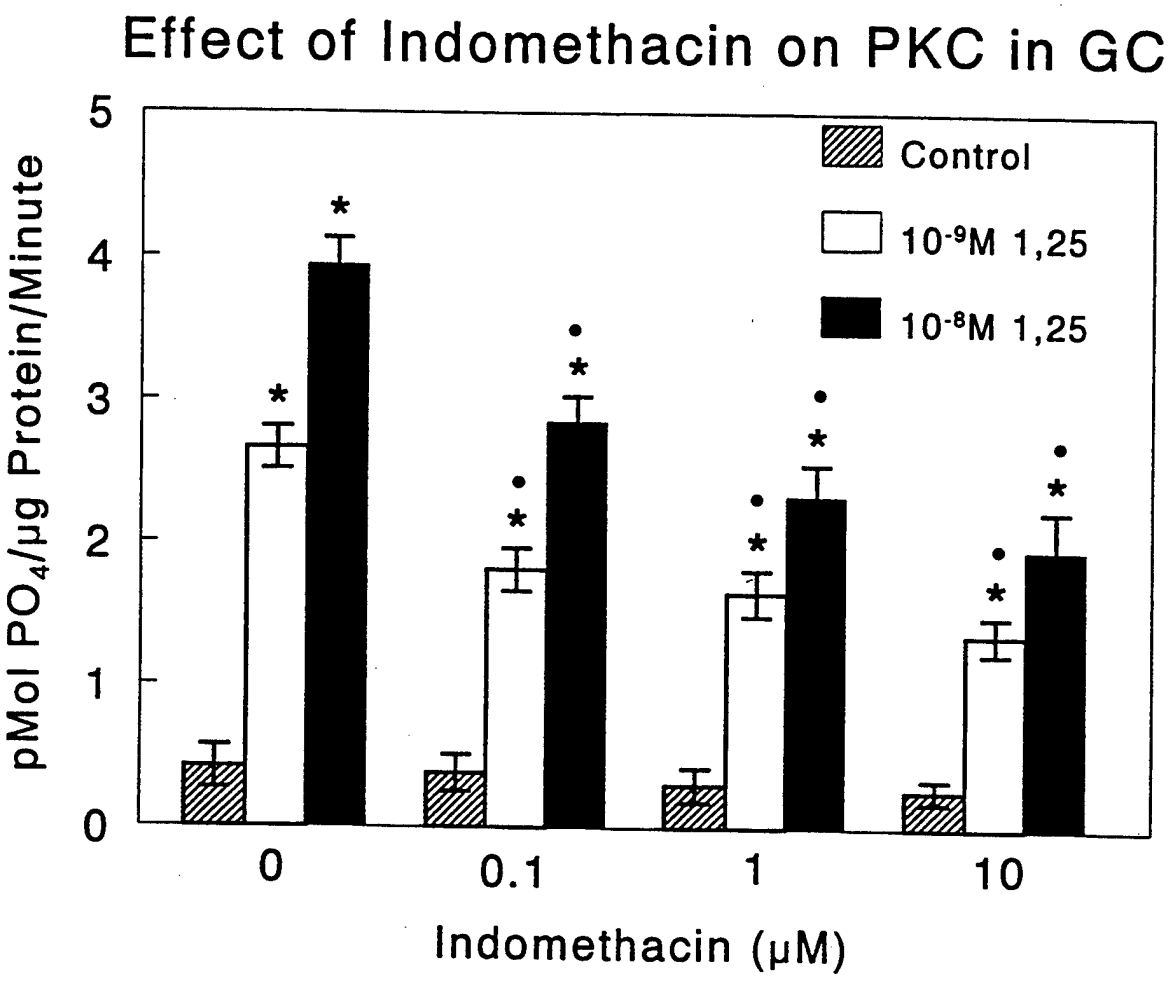
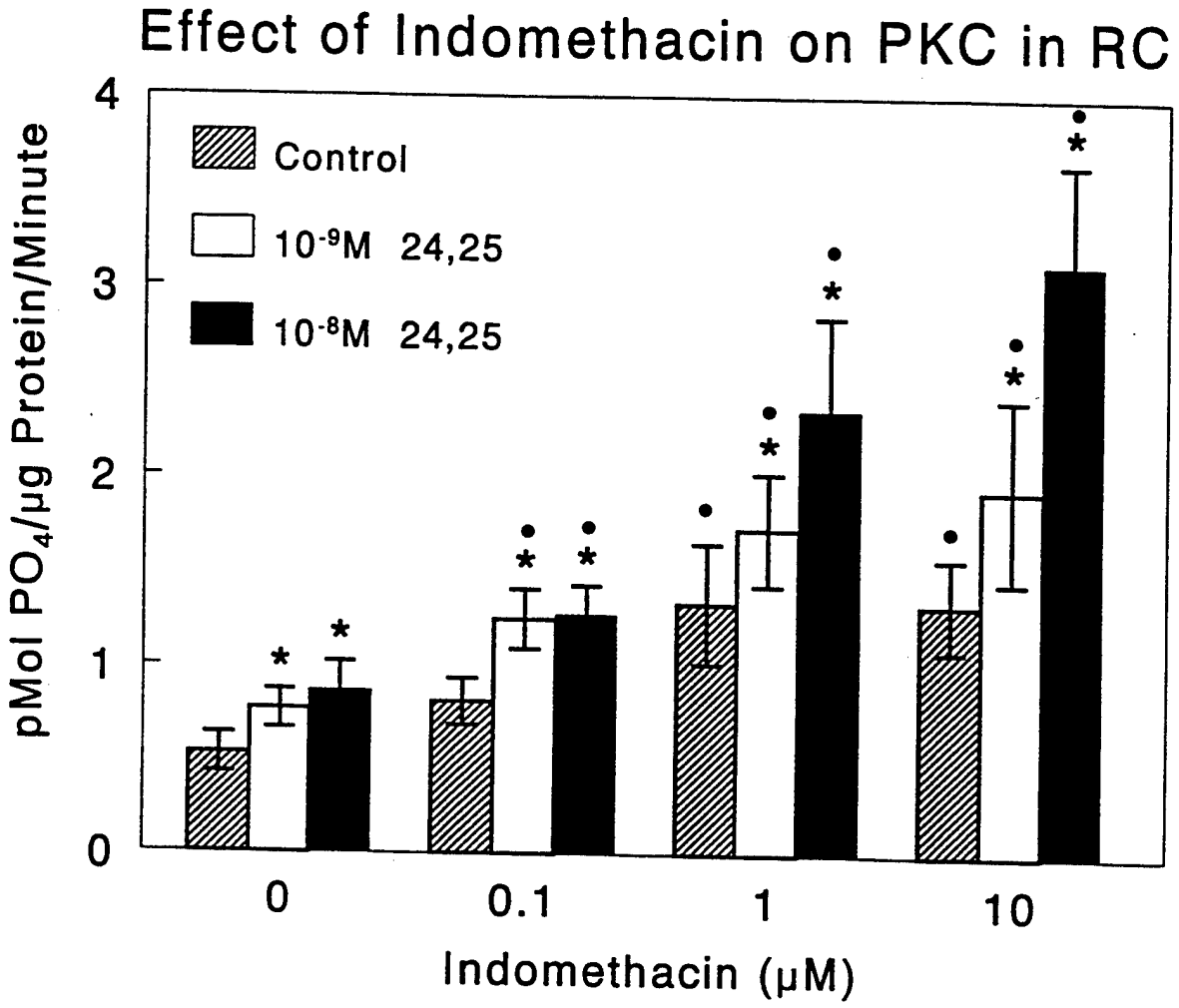

Effect of Indomethacin on PKC in GC
pMol PO4/µg Protein/Minute
0
1
2
3
4
5
Control
10-9M 1,25
10-8M 1,25
0
0.1
1
10
Indomethacin (µM)

Figure 14: Effect of indomethacin on basal and vitamin D_3 metabolite-stimulated PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cultures were treated with 0, 0.1, 1 or 10 μM indomethacin in the absence or presence of 10^{-9} M or 10^{-8} M 24,25-$(OH)_2D_3$ for 90 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean ± SEM of six cultures. Data are from one of two identical experiments. *P<0.05, vs. untreated control; °P <0.05, vs. cultures without indomethacin.


Effect of Indomethacin on PKC in RC
Control
10-9M 24,25
10-8M 24,25
pMol PO4/µg Protein/Minute
0
1
2
3
4
0
0.1
1
10
Indomethacin (µM)

cells, the 24,25-$(OH)_2D_3$-stimulated increase in PKC specific activity was significantly enhanced for every concentration of indomethacin and 24,25-$(OH)_2D_3$ tested. The addition of indomethacin at concentrations of 0.1, 1 and 10 μM stimulated PKC specific activity by 62%, 124% and 152%, respectively, in cultures treated with 10^{-9} M 24,25-$(OH)_2D_3$ and by 47%, 172% and 261%, respectively, in cultures treated with 10^{-8} M 24,25-$(OH)_2D_3$.

E. Effect of Prostaglandin E_2

There was a dose-dependent increase in PKC specific activity in growth zone cell cultures following the addition of prostaglandin E_2, a downstream metabolite of phospholipase A_2 and cyclooxygenase action (Figure 15). After 9 minutes, PKC specific activity was significantly stimulated when cells were treated with either 0.015, 0.06 or 0.23 ng/ml prostaglandin E_2. At the highest dose, 0.23 ng/ml prostaglandin E_2, PKC specific activity was increased by 123%. However, a significant increase in PKC specific activity at 90 minutes was evident only in cultures treated with 0.06 and 0.23 ng/ml prostaglandin E_2. In these cultures, the addition of 0.23 ng/ml prostaglandin E_2 resulted in only a 27% increase in PKC stimulation.

The addition of prostaglandin E_2 to resting zone chondrocyte cultures had the opposite effect on PKC specific activity. Prostaglandin E_2 treatment now resulted in a dose-dependent reduction in PKC specific activity at both 9 and 90 minutes (Figure 16). When cultures were treated with 0.015, 0.06 or 0.23 ng/ml prostaglandin E_2, PKC specific activity was significantly decreased with a greater effect evident at 90 minutes than 9 minutes. The addition of 0.23 ng/ml prostaglandin E_2 resulted in a reduction in PKC specific activity of 81% and 65% at 90 and 9 minutes, respectively.

Figure 15: Effect of prostaglandin E_2 (PGE_2) on PKC specific activity in growth zone chondrocytes. Confluent, fourth passage growth zone cultures were treated with 0.015-0.23 ng/ml PGE_2 for 9 or 90 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean $\pm$ SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control.

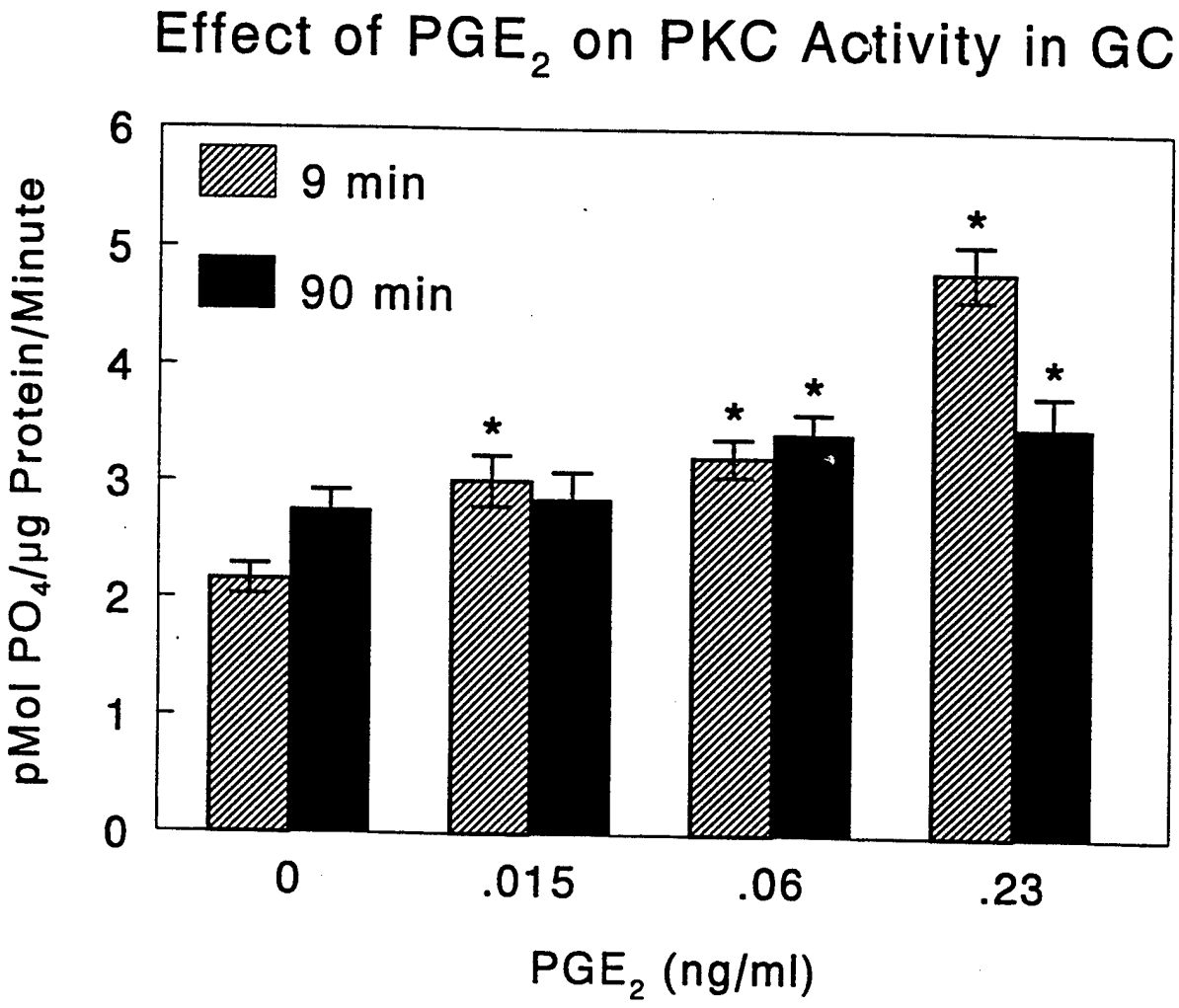
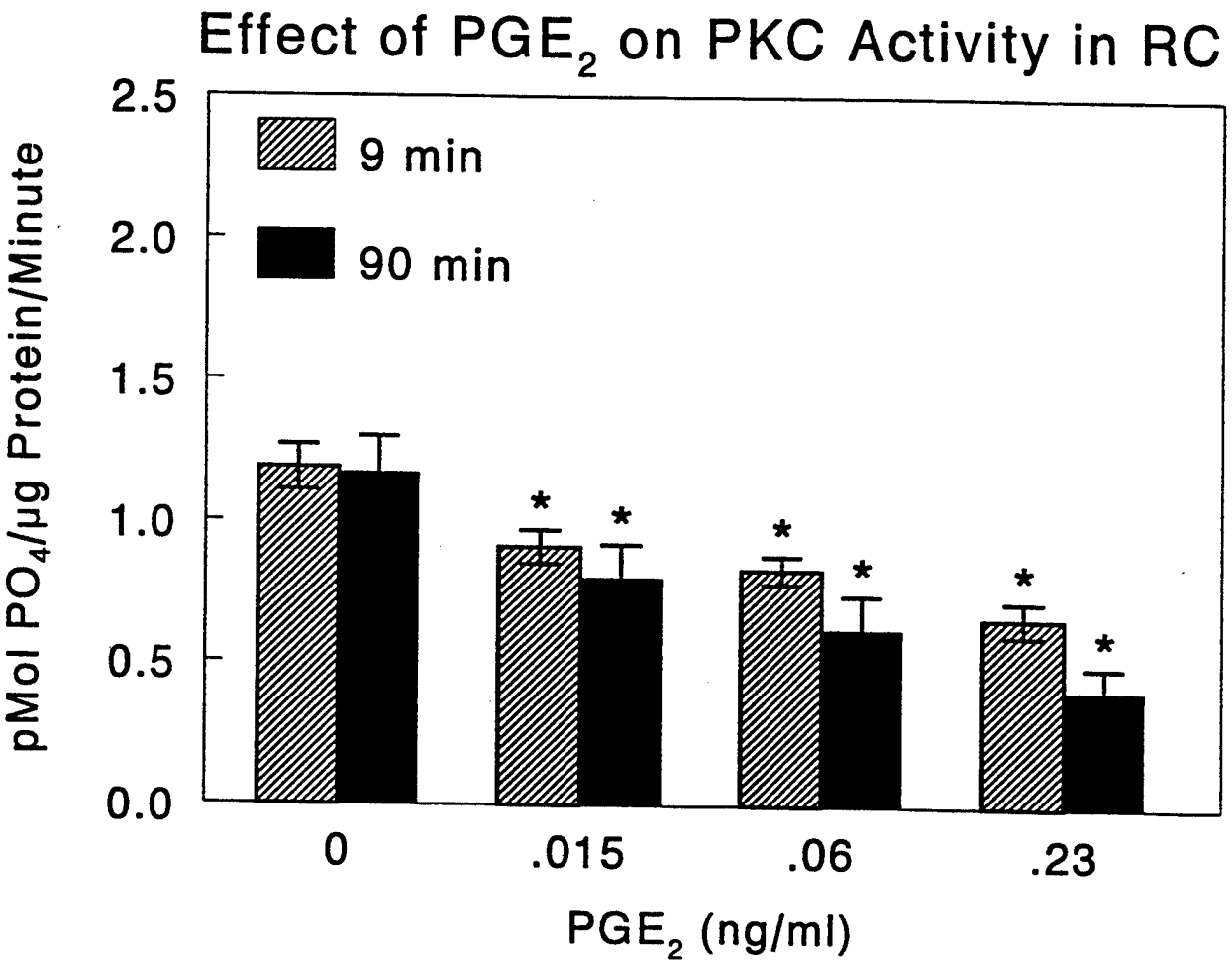

Effect of PGE2 on PKC Activity in GC
9 min
90 min
pMol PO4/µg Protein/Minute
0
1
2
3
4
5
6
*
*
*
*
*
0
.015
.06
.23
PGE2 (ng/ml)

Figure 16: Effect of prostaglandin E_2 (PGE_2) on PKC specific activity in resting zone chondrocytes. Confluent, fourth passage resting zone cultures were treated with 0.015-0.23 ng/ml PGE_2 for 9 or 90 minutes. At harvest, the cell layers were assayed for protein kinase C specific activity. Values are the mean $\pm$ SEM of six cultures. Data are from one of two identical experiments. *$P<0.05$, vs. untreated control.


Effect of PGE2 on PKC Activity in RC
9 min
90 min
pMol PO4/µg Protein/Minute
2.5
2.0
1.5
1.0
0.5
0.0
0
.015
.06
.23
PGE2 (ng/ml)

DISCUSSION AND SUMMARY

The aim of the present study was to examine the role of phospholipase A_2 and the products of its action in mediating the influence of vitamin D_3 metabolites on PKC specific activity in both growth zone and the less differentiated resting zone chondrocytes. To accomplish this, we evaluated the effects of specific inhibitors (quinacrine, OEPC, and $AACOCF_3$) and activators (melittin and mastoparan) of phospholipase A_2, as well as the downstream products of phospholipase A_2 action, arachidonic acid and prostaglandin E_2, on the cells in the presence or absence of the vitamin D_3 metabolites. In addition, we examined the effect on PKC specific activity when prostaglandin E_2 production was blocked following cyclooxygenase inhibition with indomethacin.

When the phospholipase A_2 activators (melittin or mastoparan), arachidonic acid, or prostaglandin E_2 were added to growth zone chondrocyte cultures, there was a dose-dependent increase in PKC specific activity in both control and 1,25-$(OH)_2D_3$-treated cultures. By contrast, the addition of melittin, mastoparan, arachidonic acid, or prostaglandin E_2 to resting zone cell cultures resulted in a dose-dependent reduction in PKC specific activity in both control and 24,25-$(OH)_2D_3$-treated cultures. The opposite effects were seen in both cell types when phospholipase A_2 activity was inhibited with quinacrine, OEPC, or $AACOCF_3$. Moreover, while the addition of indomethacin partially blocked the 1,25-$(OH)_2D_3$-dependent PKC specific activation in growth zone chondrocytes, it stimulated the 24,25-$(OH)_2D_3$-induced PKC specific activity in resting zone chondrocytes. In general, when any of the experimental reagents were added either alone or to vitamin D_3 treated cultures, maximal PKC specific activation or inhibition was time-dependent and consistent with previously reported vitamin D_3 cell maturation-dependent PKC specific activity

(Sylvia et al., 1993). For growth zone and resting zone cell cultures, this effect was observed at 9 minutes and 90 minutes, respectively. Taken together, our results suggest that PKC specific activity is at least partially regulated via phospholipase A_2 stimulation in growth zone cells and phospholipase A_2 inhibition in resting zone cells in a time- and dose-dependent manner.

More specifically, in growth zone chondrocytes, the addition of the phospholipase A_2 inhibitors resulted in a reduction in both basal and 1,25-$(OH)_2D_3$-induced PKC specific activity. Evidence suggests that both secretory and cytosolic phospholipase A_2 are involved since OEPC, an inhibitor of the secretory form (Magolda and Galbraith, 1989), and $AACOCF_3$, an inhibitor of the cytosolic form (Street et al., 1993), blocked the 1,25-$(OH)_2D_3$-dependent stimulation of PKC. By contrast, the opposite effect was observed in resting zone chondrocytes. While the addition of the phospholipase A_2 inhibitors alone caused a dose-dependent increase in PKC specific activity, the combination of the phospholipase A_2 inhibitors with 24,25-$(OH)_2D_3$ resulted in a synergistic activation of PKC. Again, both the secretory and cytosolic forms of phospholipase A_2 appear to be involved since the addition of either OEPC or $AACOCF_3$ enhanced the 24,25-$(OH)_2D_3$-induced PKC specific activity.

When phospholipase A_2 activity was stimulated by melittin or mastoparan, the effect on PKC specific activity was again cell-maturation specific. In growth zone chondrocytes, the addition of either phospholipase A_2 activator caused significant, dose-dependent increases in basal PKC specific activity, and when added to cells with 1,25-$(OH)_2D_3$, elicited a synergistic increase in activity. By contrast, both basal and 24,25-$(OH)_2D_3$-induced PKC specific activity was dose-dependently reduced following the addition of the phospholipase A_2 activators to resting zone chondrocyte cultures.

Arachidonic acid, the product of phospholipase A_2 action, increased PKC specific activity and synergistically stimulated the action of 1,25-$(OH)_2D_3$ on growth zone chondrocyte PKC. Since arachidonic acid can modulate new gene expression via RXR receptors (Bocos et al., 1995), this may explain the synergistic increase in PKC specific activity evident in cultures treated with arachidonic acid in combination with 1,25-$(OH)_2D_3$. The fact that low levels of arachidonic acid are not synergistic with 1,25-$(OH)_2D_3$, whereas high levels are (Sylvia et al., 1998), suggests a threshold effect indicative of a concerted action involving new gene expression. This interpretation is supported in this study by the fact that while the effect on PKC specific activity following the addition of arachidonic acid with 1,25-$(OH)_2D_3$ was additive at 9 minutes, it was synergistic at 90 minutes. By contrast, the addition of arachidonic acid to resting zone cell cultures inhibited PKC specific activity and partially blocked in a dose-dependent manner the action of 24,25-$(OH)_2D_3$ on resting zone chondrocyte PKC.

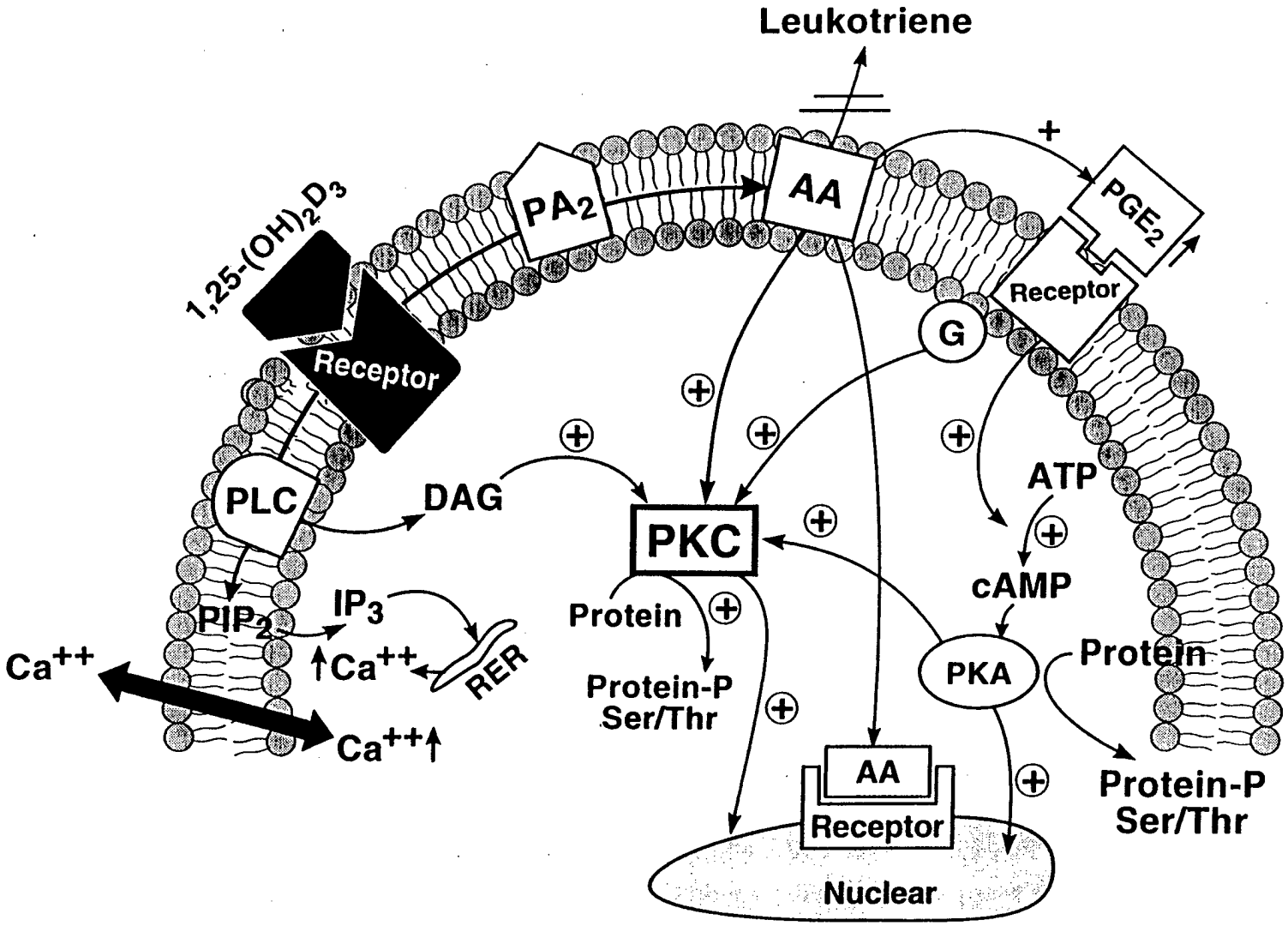
The vitamin D_3 metabolites regulate in a time-dependent manner the concentration of arachidonic acid formed by phospholipase A_2 action on membrane phospholipids (Swain et al., 1992). The newly formed arachidonic acid is subsequently metabolized by cyclooxygenase or lipoxygenase, resulting in prostaglandin or leukotriene production, respectively. Indomethacin, a cyclooxygenase-1 inhibitor, blocks the conversion of arachidonic acid to prostaglandin. Our research demonstrated that at least part of the cell maturation-dependent effects of arachidonic acid are mediated through its metabolism to prostaglandin. In growth zone chondrocytes, this was supported by the observation that inhibition of prostaglandin E_2 formation blocked the stimulation of PKC by arachidonic acid alone, and decreased the synergistic effects of arachidonic acid with 1,25-$(OH)_2D_3$. Moreover, the addition of prostaglandin E_2 caused a rapid dose-dependent increase in PKC specific activity with maximal stimulation occurring at 9 minutes. By

contrast, inhibition of prostaglandin E_2 formation in resting zone chondrocytes dose-dependently enhanced the basal and 24,25-$(OH)_2D_3$-induced PKC specific activity. Finally, the addition of prostaglandin E_2 caused a time- and dose-dependent reduction of PKC specific activity with the maximal effect observed at 90 minutes.

Our results indicate that the vitamin D_3 metabolite-induced changes in phospholipase A_2 activity are directly related to changes in PKC specific activity and that this effect is cell maturation- and time-dependent, and metabolite-specific. Moreover, our study indicates that the effect of vitamin D_3 metabolites on phospholipase A_2 is mediated by the release of arachidonic acid, and subsequently, the formation of prostaglandin E_2. Although others have demonstrated that arachidonic acid can act directly on cells to mediate their function (Lopez-Ruiz et al., 1992; Giaume et al., 1997), the mechanisms involved in the arachidonic acid mediation of PKC specific activity are poorly understood. Initially, it is likely that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ interact directly with phospholipase A_2. This early interaction results in the differential production of arachidonic acid as a function of time and cell maturation (Swain et al., 1992). Whereas 1,25-$(OH)_2D_3$ stimulates arachidonic acid release by growth zone cells within 5 minutes, 24,25-$(OH)_2D_3$ does not cause an increased release in resting zone cells for 15 minutes. As evidenced by synergistic increases in PKC specific activity, the initially rapid effects of arachidonic acid may be amplified at later time points in a genomic fashion (Bocos et al., 1995).

The rapid, but cell maturation-specific effects on phospholipase A_2 activity following exposure to the vitamin D_3 metabolites may be accounted for not only by differences in membrane lipid composition and fluidity (Boyan et al., 1988; Swain et al., 1993), but also from the existence of specific vitamin D_3 membrane receptors. Recently, 1,25-$(OH)_2D_3$ receptors comparable to those identified in chick intestinal epithelial cells (Nemere et al., 1994; Norman et al., 1994) were

Mechanism of Action of $1,25\text{-}(OH)_2D_3$



Growth Zone Chondrocytes

Sylv5-1/98

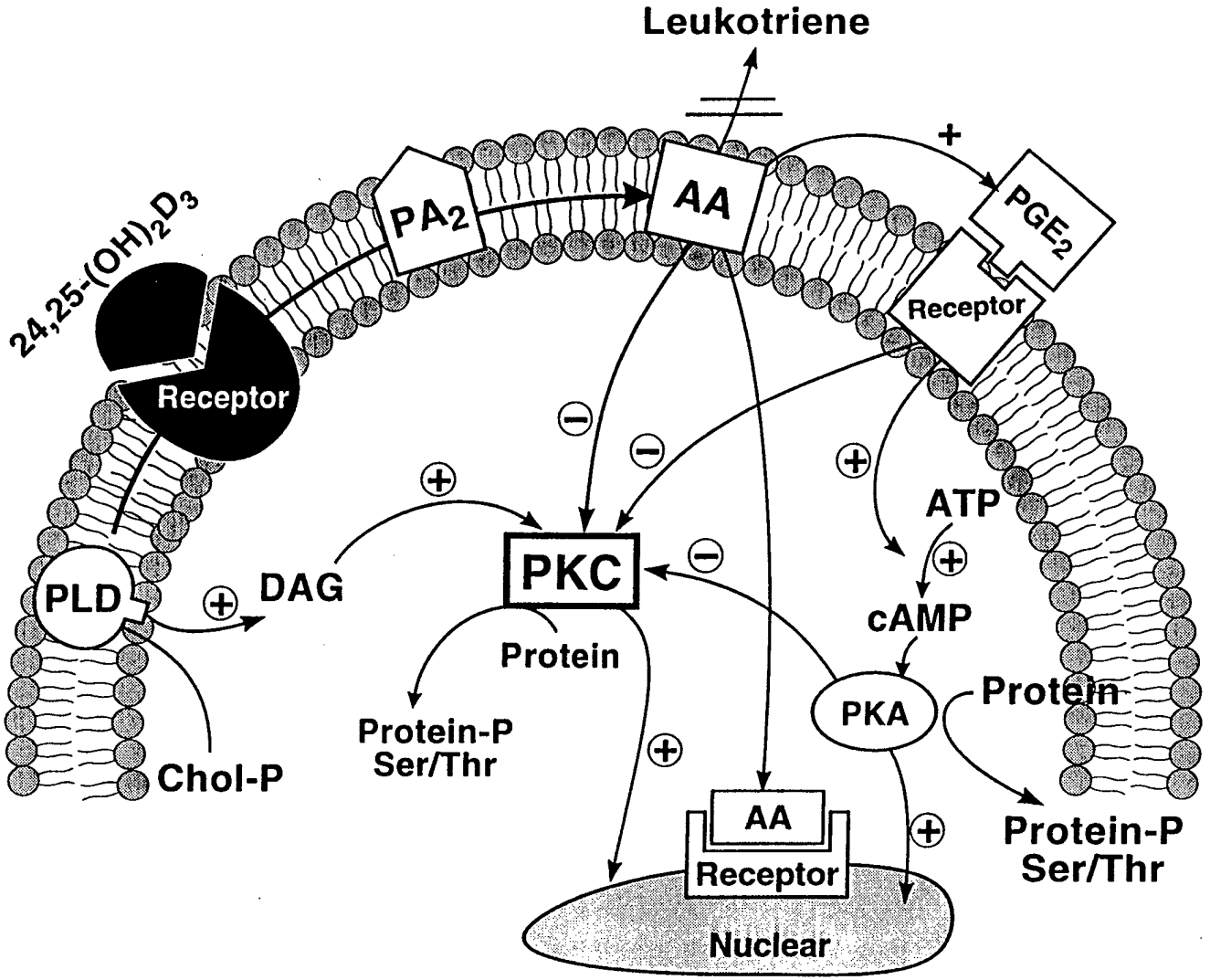
Figure 17

isolated in growth zone and resting zone chondrocytes (Nemere et al., 1997). Substantial evidence also supports the existence of specific receptors for 24,25-$(OH)_2D_3$ in other systems (Takeuchi and Guggino, 1996; Sundell and Bjornsson, 1990). Ongoing studies in the laboratory using A-ring and hybrid analogues indicate that membrane receptors exist in the costochondral chondrocytes (Boyan et al., 1997a, Greising et al., 1997; Schwartz et al., 1997) that regulate PKC specific activity in a metabolite-specific manner.

Our results, when taken in the context with previous work, provide new insights into the complex mechanisms involved in the vitamin D_3 metabolite-specific activation of PKC. In growth zone chondrocytes, 1,25-$(OH)_2D_3$ regulates PKC specific activity by two distinct phospholipid-dependent mechanisms: production of DAG via phospholipase C (Sylvia et al., 1993; Sylvia et al., 1998) and production of arachidonic acid via phospholipase A_2 stimulation. By contrast, the 24,25-$(OH)_2D_3$ regulation of PKC specific activity in resting zone cells proceeds through two different phospholipid-dependent pathways. In these cell cultures, 24,25-$(OH)_2D_3$ regulates PKC specific activity by: production of DAG via phospholipase D (Helm et al., 1996) and inhibition of the production of arachidonic acid and its downstream product, prostaglandin E_2, via phospholipase A_2 inhibition.

To summarize, a proposed mechanism for the rapid action of 1,25-$(OH)_2D_3$ on growth zone chondrocytes is presented in *Figure 17*. Following the initial binding of 1,25-$(OH)_2D_3$ to the growth zone cell membrane receptor, phospholipase A_2 activity is increased (Schwartz and Boyan, 1988a). This activity results in arachidonic acid turnover (Schwartz et al., 1990) and production of prostaglandin E_2 (Schwartz et al., 1992a). Ultimately, there is a change in membrane fluidity (Swain et al., 1993) and Ca^{2+} flux (Langston et al., 1990; Schwartz et al., 1991). The binding of 1,25-$(OH)_2D_3$ also stimulates DAG production via phospholipase C. This

Mechanism of Action of 24,25-$(OH)_2D_3$



Resting Zone Chondrocytes

Sylv4-1/98

Figure 18

mechanism involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent generation of the second messengers, inositol 1,4,5-trisphosphate and DAG (Helm et al., 1996). The presence of inositol 1,4,5-trisphosphate leads to higher intracellular Ca^{2+} levels via stimulated endoplasmic reticulum release and increased influx from the extracellular fluid (Berridge, 1987). DAG is an important allosteric activator of PKC (Hug and Sarre, 1993). Our research has demonstrated that stimulation of arachidonic acid release also activates PKC. In addition to its effect of PKC, increased arachidonic acid production also stimulates the production of prostaglandin E_2, an important regulator of chondrocytes. The stimulation of prostaglandin E_2 activates the G-protein pathway, activating adenylate cyclase, cyclic AMP, and protein kinase A, independent of tyrosine kinase (Helm et al., 1996). PKC specific activation, whether from phospholipase C or phospholipase A_2 stimulation, results in a signal transduction cascade caused by the phosphorylation of serine and threonine residues. The end result is mitogen-activated protein kinase activation, phosphorylation of AP-1, and increased transcription relevant gene promoters (Boyan et al., 1997a). Ultimately, PKC signal transduction is essential for the cellular processes involved in endochondral bone formation (Boyan et al., 1997a).

The proposed mechanism for the rapid action of 24,25-$(OH)_2D_3$ on resting zone chondrocytes is different than 1,25-$(OH)_2D_3$ on growth zone chondrocytes and is shown schematically in *Figure 18*. Although the sequence of activity initially involves the binding of 24,25-$(OH)_2D_3$ to the resting zone cell membrane, the end result is phospholipase A_2 inhibition (Schwartz and Boyan, 1988a) rather than activation. This effect changes fatty acid turnover (Schwartz et al., 1990; Swain et al., 1992), release of arachidonic acid (Schwartz et al., 1990; Swain et al., 1992), and prostaglandin production (Schwartz et al., 1992a). There is a resultant change in membrane fluidity (Swain et al., 1993) and Ca^{2+} flux (Langston et al., 1990; Schwartz et

al., 1991). Similar to 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ also stimulates PKC specific activity through increased DAG production (Helm et al., 1996), but the mechanism of action is different. Although DAG production is increased, the process does not involve phospholipase C (Helm et al., 1996). This suggests the increased levels of DAG are a result of phosphatidylcholine metabolism via phospholipase D. PKC stimulation is again tyrosine kinase independent (Helm et al., 1996). The present research demonstrates that PKC specific activity can also be stimulated via phospholipase A_2 pathway inhibition. By contrast, the addition of prostaglandin E_2 inhibits PKC specific activity through protein kinase A. Again, PKC dictates cellular behavior through protein phosphorylation.

The research in this study at the cellular level is necessary in order to provide the required foundation for future clinical applications. The manipulation of any cellular function in an attempt to regulate an anabolic process can only be accomplished after thoroughly understanding the complex mechanisms involved. The goal is to take an established *in vitro* finding and apply it to an *in vivo* situation to achieve a desired therapeutic endpoint. As a periodontist, the ultimate goal is the complete regeneration of the lost periodontium, including alveolar bone, cementum, periodontal ligament, and gingival attachment.

Promising new developments in the field of periodontal regeneration involve the use of bone morphogenetic proteins (BMPs). BMPs are osteoinductive materials which initiate the development of tissues and organ systems by stimulating undifferentiated cells to convert phenotypically (Urist, 1994). Of the nine BMPs thus far reported in the literature, eight of these, BMP-2 through BMP-9, are related to one another. Due to their amino acid sequences, the same BMPs are classified as belonging to the TGF-β superfamily (Lee, 1997). Except for BMP-1, all other BMPs are capable of inducing bone formation. BMPs are the only known molecules

capable of forming cartilage and bone in an ectopic site (Lee, 1997; Zhang et al., 1997; Reddi and Anderson, 1976). Since endochondral bone formation is involved in both ectopic bone formation (Zhang et al., 1997) and periodontal regeneration (Amar et al., 1997), BMPs may provide the impetus necessary for enhanced results. In fact, encouraging osteogenic results have been reported following the use of various BMPs for maxillary sinus floor augmentation (Boyne et al., 1997), mandibular molar grade III furcation regeneration (Giannobile et al., 1998), and osseous regeneration around dental implants (Cochran et al., 1997).

In the endochondral pathway, bone morphogenetic protein induces chondrocyte differentiation and matrix mineralization, the same effects that the vitamin D_3 metabolites regulate. The present research has demonstrated that vitamin D_3 metabolites regulate these cellular functions via PKC specific activation in a cell maturation-dependent and metabolite-specific manner. By combining two anabolic factors involved in endochondral bone formation, namely BMP and vitamin D_3, the possibility of an additive or synergistic increase in osteogenic potential exists. In fact, preliminary data in our laboratory using a related growth factor supports this concept. Specifically, the combination of 24,25-$(OH)_2D_3$ and TGF-β synergistically increased alkaline phosphatase production, a marker for bone mineralization (Schwartz et al., 1998).

To further enhance the osteogenic potential of the BMP and vitamin D_3 combination, the present experiments support the need to regulate PKC specific activity. The selective stimulation of PKC activity in accordance with the level of cell maturation should aid in the regulation of bone and cartilage formation. The research presented in this laboratory, as well as others, have demonstrated this is possible because PKC specific activity can be stimulated in a cell maturation-dependent and metabolite-specific manner by adding various activators or inhibitors of phospholipase A_2, phospholipase C, or phospholipase D.

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VITA

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