EFFECT OF TRANSFORMING GROWTH FACTOR BETA (TGFβ) AND VITAMIN D_3 METABOLITES ON PROTEIN KINASE C MEDIATED SIGNAL TRANSDUCTION IN RAT COSTOCHONDRAL CHONDROCYTE CULTURES

A
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DEDICATION

I dedicate this thesis to the memory of my father Dr. Harold R. Mackey. His love for dentistry and dedication to the best possible care have become my aspiration.

This thesis would not have been possible without the love, patience and encouragement of my wife Sharon and my three adorable children Joshua, Jessica, and Jennie. It was there sacrifice and devotion that saw me through.

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I am deeply grateful for the friendship and guidance of Drs. Barbara Boyan and Zvi Schwartz who made this research endeavor possible. My daily association with Dr. Victor Sylvia was a joy and made my laboratory experience pleasurable as well as scientifically stimulating. It is to him that I am most thankful, for his time and knowledge in answering many a question and will always count as a close friend. The scholarly advice of Dr. David Dean was indispensable in the writing of this manuscript. I also am thankful for Reuben Gomez and Monica Luna for their laboratory expertise.

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It is known that vitamin D_3 metabolites and transforming growth factor β (TGF-β) regulate proliferation and differentiation of chondrocytes isolated from the growth zone or resting zone of rat costochondral cartilage. It was hypothesized that both TGF-β and the vitamin D metabolites play a role in signal transduction in the rat costochondral chondrocyte model and that the regulation of protein kinase C by these metabolites would be cell maturation-dependent. Confluent, fourth passage cultures of growth zone and resting zone chondrocytes were treated with vitamin D_3 metabolites for up to 24 hours, lysed, and the cell extracts assayed for protein kinase C (PKC) specific activity using a specific peptide substrate. The addition of 1,25-$(OH)_2D_3$ to growth zone chondrocyte cultures resulted in a rapid, dose-dependent stimulation of PKC specific activity. The effect was significant at 10^{-9}-10^{-7}M 1,25-$(OH)_2D_3$ and began 3 minutes after addition of hormone and continued for an additional 90 minutes. In contrast, 1,25-$(OH)_2D_3$ had no effect on PKC specific activity in resting zone

chondrocyte cultures. The addition of 24,25-$(OH)_2D_3$ to resting zone chondrocyte cultures also produced PKC activation. The response was significant at 10^{-8}-10^{-7}M 24,25-$(OH)_2D_3$ and slower than that seen for 1,25-$(OH)_2D_3$ and the growth zone cells. Increases in PKC specific activity were detectable 90 minutes after addition of hormone and lasted up to 360 minutes. 24,25-$(OH)_2D_3$ had no effect on PKC specific activity in cultures of growth zone cells at all times and concentrations examined. Pretreatment of the cultures with U73122, a phospholipase C inhibitor, decreased 1,25-$(OH)_2D_3$-stimulated PKC specific activity, but had no effect upon 24,25-$(OH)_2D_3$-induced activity. The tyrosine kinase inhibitor, genistein, did not inhibit the PKC specific activity response in cultures treated with either of the vitamin D_3 metabolites. Neither actinomycin D nor cycloheximide affected 1,25-$(OH)_2D_3$-induced PKC specific activity in growth zone chondrocyte cultures, while both compounds inhibited 24,25-$(OH)_2D_3$-induced activity in resting zone chondrocyte cultures. The results of this portion of the study indicate that vitamin D metabolites stimulate PKC specific activity in a metabolite- and cell-maturation-specific manner. Effects of 1,25-$(OH)_2D_3$ appear to be nongenomic, whereas the effects of 24,25-$(OH)_2D_3$ probably involve a genomic mechanism.

In contrast, addition of transforming growth factor-beta (TGF-β) to cultures of both resting zone and growth zone chondrocytes produced dose-dependent increases in PKC specific activity. Significant effects on enzyme activity were observed 6 hours after addition of growth factor with maximal stimulation found at 12 hours; baseline levels of activity returned after 24 hours. Neither the phospholipase C inhibitor, U73122, nor the tyrosine kinase inhibitor, genistein, significantly reduced the response to TGFβ. However, treatment of cultures with actinomycin D or cycloheximide prior to addition of TGFβ resulted in a dose-dependent inhibition of TGFβ-stimulated PKC specific activity in both growth zone and resting zone chondrocytes. The time-course of activation and insensitivity to U73122 suggest that phospholipase C-mediated events are not involved. Similarly, since genistein has no effect, tyrosine kinases are probably not involved in TGFβ-induced PKC specific activity in

chondrocytes. Rather, the reduction in PKC specific activity observed when TGFβ is administered along with actinomycin D or cycloheximide indicates that new gene expression and protein synthesis are required for the response. Because an earlier and more prolonged response was seen in resting zone chondrocytes than in growth zone chondrocytes, the effect of TGFβ on PKC specific activity appears to be cell maturation-dependent. These results suggest that the effect of TGFβ on chondrocytes may be mediated by PKC. However, since the effect is very slow, it may require new PKC production. Moreover, the classic mechanism for activation of PKC by phospholipase C was not found, suggesting a novel mechanism of activation.

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I. INTRODUCTION

A. Signal Transduction And Protein Kinase C:

Communication between the extracellular and intracellular compartments is critical to normal functioning of cells, tissues and the entire organism. The biochemical pathways used for the transduction of signals into the cell has been an area of considerable research. The elucidation of signal transduction pathways used by chondrocytes and osteoblasts will have significant implications in cartilage repair, fracture healing, periodontal regenerative procedures, and endosseous implants. This thesis will examine the signal transduction pathways utilized by rat costochondral chondrocytes in response to treatment with vitamin D_3 metabolites and transforming growth factor-beta (TGF-β).

Hormones and peptide growth factors influence their target cells by binding to specific receptors in the plasma membrane (Felig *et al.*, 1981). Sutherland *et al.*, (1966) suggested that the cell membrane serves as the site of action for hormones. They observed that the interaction of catecholamines with the plasma membrane of pigeon erythrocytes led to the activation of adenylate cyclase. Sutherland's concept of hormone action, the first messenger, stimulating cAMP or some other mediator as a second (intracellular) messenger is central to the study of signal transduction. Other membrane enzymes, regulated by binding of primary messengers, are involved in inositol turnover, sodium-potassium ATPase activity, membrane-bound protein kinases, methyltransferases involved in phospholipid methylation, and phospholipases responsible for deacylation of membrane phospholipids. Membrane phospholipids provide precursors, such as arachidonic acid, for prostaglandin, thromboxane, and prostacyclin biosynthesis which are important inflammatory mediators. Thus, there are numerous pathways by which a hormone or growth factor could influence intracellular metabolism (Felig *et al.*, 1981).

Protein kinases are enzymes that catalyze the transfer of phosphate groups from one compound to another (Lehninger, 1982). Protein kinase C (PKC) has been intensively studied because its believed to be involved in signal transduction and, possibly, tumorigenesis. Originally identified in rat brain extracts as a calcium and phospholipid activated protein kinase (Takai, *et al.*, 1979), it was ultimately linked to signal transduction because diacylglycerol, one of the early products of inositol phospholipid breakdown, greatly increased the affinity of PKC for Ca^{+2} and its activity (Takai *et al.*, 1979).

Evidence that activation of PKC was linked to signal transduction has primarily come from experiments with platelets (Kawahara *et al.*, 1980). Kawahara *et al.*, showed that platelets, when stimulated, produce diacylglycerol containing arachidonic acid, and that this reaction is accompanied by the disappearance of inositol phospholipids. This appearance of diacylglycerol in membranes was found to always be associated with the activation of PKC.

Several phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are potent tumor promoters. Many studies, with various cell types, suggest that phorbol esters act on the cell membrane surface (Blumberg, 1980). Studies by Castagna *et al.*, (1982) have provided evidence that PKC is a target for phorbol esters since these agents directly activate this enzyme. Like diacylglycerol, TPA increases the affinity of PKC for Ca^{2+}, resulting in enzyme activation.

Currently, ten isoforms of PKC have been identified. Four isoforms (alpha, beta-I, beta-II and gamma), called the classical protein kinases, were identified by screening complementary cDNA libraries. Subsequently, six other isoforms (delta, epsilon, lambda, nu, theta, and zeta) have been identified. In rat, gamma and epsilon isoforms are found solely in brain and spinal cord, whereas beta-I, beta-II and delta isoforms are found in numerous organ systems. The alpha isoform of PKC is the most widely distributed. This enzyme consists of a single polypeptide chain with a molecular weight of 77 kDa. All isoforms of PKC phosphorylate proteins on serine and threonine residues (Nishizuka, 1984).

PKC isoforms alpha, beta-I, beta-II and gamma have been shown to be Ca^{2+}/phospholipid/diacylglycerol (DAG)-dependent protein kinases (Knopf *et al.*, 1986, Kikkawa *et al.*, 1987). PKC activation is thought to be involved in a variety of cell responses, including cell proliferation, gene expression, membrane transport, and the excretion of hormones and neurotransmitters (Nishizuka, 1984). While these isoforms constitute a family, studies show that there are functional differences between the members. These isoforms would not be expected to function interchangeably, but to act in parallel to transduce specific signals to the cell (Parker *et al.*, 1989).

van Leeuwen *et al.*, (1992) have shown that 1,25-$(OH)_2D_3$ stimulates osteocalcin synthesis by osteoblast-like cells and that this was inhibited 30-70% in the presence of PKC inhibitors. These results showed for the first time, that PKC is involved in mediating the effect of 1,25-$(OH)_2D_3$ on bone cells. Obeid *et al.*, (1990) treated human promyelocytic leukemia cells with 1,25-$(OH)_2D_3$ and found that mRNA for both PKC alpha and beta was increased. This was the first report of transcriptional activation of PKC, and may be a mechanism for the long term alteration of the PKC pathway. Yada *et al.*, (1989) found that only 1-alpha-25-$(OH)_2D_3$ induced the formation of a mature envelope in murine keratinocytes. Inhibition of PKC by pretreatment with H-7, a specific inhibitor of PKC, caused significant suppression of the vitamin D_3 affect. These findings suggest that PKC is involved in mediating the effect of vitamin D metabolites on these cells.

Ohtsuki *et al.*, (1992) demonstrated that transforming growth factor-β (TGFβ) induced expression of transcription factors and prevented phosphorylation of the retinoblastoma susceptibility gene product. These responses were blocked by the protein kinase C inhibitors H7, H8 and H9. Wrenn *et al.*, (1993) investigated the role of PKC in the action of TGF-β on cultured embryonic avian vascular smooth muscle from different tissues. A fifteen minute exposure to TGF-β significantly increased PKC activity in these cells. TGF-β also increased the production of diacylglycerol (DAG) as early as 15 seconds after addition. DAG is

considered to be a second messenger molecule responsible for activation of PKC. Chakrabarty (1992) showed that induction of carcinoembryonic antigen by TGF-β1 was associated with PKC activation. Treatment of cells with the PKC inhibitors calphostin C or H-7 blocked activation of the carcinoembryonic antigen as well as PKC activity. The diacylglycerol kinase inhibitor, R59 022, and the G-protein inhibitors cholera toxin and pertussis toxin had no affect. It was concluded that TGF-β1 regulates carcinoembryonic antigen expression through a pathway that utilizes PKC. These studies strongly suggest a significant role for PKC in TGF-β-mediated signal transduction.

B. Endochondral Ossification and Vitamin D Metabolites:

Endochondral bone formation involves the differentiation of mesenchymal stem cells into chondrocytes and calcification of the cartilaginous matrix they form. Long bone growth, fracture repair and bone induction all require endochondral ossification and therefore calcification of cartilage is critical (Glowacki, 1982; Ksiazek and Moskalewski, 1983). During long bone development the cartilaginous epiphysis is separated from the diaphysis by the epiphyseal growth plate. Chondrocytes in the growth plate proliferate, mature, hypertrophy and calcify their extracellular matrix (Boskey, 1981, Anderson, 1969). Rat costochondral chondrocyte cultures have been used to understand the differentiation and regulation of cartilage cells during endochondral ossification (Boyan *et al.*, 1992). These cultures have proven to be an excellent model for studying the genomic and nongenomic effects of vitamin D metabolites, such as 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, on resting zone and growth zone chondrocytes (Boyan *et al.*, 1988; Langston *et al.*, 1990; Schwartz *et al.*, 1989; Swain *et al.*, 1992).

Chondrocytes of the proliferative zone contain extracellular matrix vesicles that are filled with a granular material (Boskey, 1981). These matrix vesicles appear to be involved in the calcification of cartilage (Anderson, 1969, 1976). Initially, crystals can be observed along the inner surface of the matrix vesicle membrane. The matrix vesicle then ruptures and the

mineral is directly deposited into the matrix (Brighton and Hunt, 1976). The vesicles are derived from the plasma membrane of the chondrocyte (Cecil and Anderson, 1978), and contain numerous enzymes which participate in mineralization, either by promoting crystal formation, or by modifying the extracellular matrix (Boyan, 1988).

Vitamin D has been shown to be essential for proper endochondral ossification (Raisz and Kream, 1983a and b). Vitamin D, or cholecalciferol, is made in the skin from an inactive form, 7-dehydrocholesterol, by reactions initiated by sunlight. Vitamin D is hydroxylated first in the liver and then in the kidney to an active form, 1,25-dihydroxycholecalciferol, which is important for calcium and phosphate metabolism (Lehninger, 1982). In vitamin D deficient chicks the growth zone of the cartilage fails to mineralize. When given vitamin D (Atkin *et al.*, 1985), or when given calcium (Balsan *et al.*, 1986), the growth plate rapidly heals.

The response of bone and cartilage cells to vitamin D metabolites is a function of their state of differentiation. 1,25-dihydroxyvitamin D_3 exerts specific effects on the alkaline phosphatase activity of osteosarcoma cells (Majeska and Rodan, 1982), human bone-derived osteoblastic cells (Beresford *et al.*, 1986), and rat calvaria (Canalis and Lian, 1985) which are dependent on the state of cell maturation. 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 exert opposing effects on alkaline phosphatase activity in chick growth plate chondrocytes (Hale *et al.*, 1986). As alluded to above, rat costochondral chondrocytes have been used to understand the differentiation and regulation of cartilage cells during endochondral ossification (Boyan *et al.*, 1992). Using this model, Schwartz *et al.* (1989), studied the effect of vitamin D metabolites on collagen synthesis and cell proliferation. They showed that addition of 1,25-$(OH)_2D_3$ to growth zone chondrocytes increased collagen synthesis, but decreased collagen synthesis in resting zone cells. The addition of 24,25-$(OH)_2D_3$ decreased collagen synthesis in resting zone chondrocytes and had no effect on growth zone cells. 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ both inhibited (^{3}H)-thymidine incorporation in resting and growth zone chondrocytes, which correlated with a decrease in

cell number. Langston *et al.* (1990), showed that calcium flux in chondrocyte cultures treated with either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ was cell maturation dependent. Schwartz *et al.* (1988a, 1988b), also showed a differential response by growth zone and resting zone chondrocytes to the addition of 1,25-$(OH)_2D_3$. In growth zone cultures, alkaline phosphatase and phospholipase A_2 specific activities were increased, whereas in resting zone cultures, this form of vitamin D had no effect on alkaline phosphatase or phospholipase A_2 specific activity. Schwartz *et al.* (1993), showed that 1,25-$(OH)_2D_3$ stimulated prostaglandin E_2 (PGE_2) production by growth zone cells in a dose-dependent manner, but had no effect on PGE_2 production by resting zone cells. In contrast, 24,25-$(OH)_2D_3$ inhibited PGE_2 production in resting zone cells and had no effect on growth zone cells.

The effect of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have also been measured in isolated matrix vesicles and compared with those of the plasma membrane isolated from rat costochondral chondrocyte cultures (Schwartz *et al.*, 1988). 1,25-$(OH)_2D_3$ was found to stimulate alkaline phosphatase and phospholipase A_2 specific activities in matrix vesicles produced by growth zone chondrocytes, but had no effect on the plasma membrane fraction, or on the matrix vesicles or plasma membranes isolated from resting zone chondrocyte cultures. 24,25-$(OH)_2D_3$, however, stimulated alkaline phosphatase activity in matrix vesicles from resting zone chondrocytes, but had no effect on plasma membranes isolated from the same cells. Growth zone chondrocyte derived matrix vesicle or plasma membrane enzymes were unaffected by 24,25-$(OH)_2D_3$. Glaser and Conrad (1981) demonstrated that matrix vesicles from the growth zone of chick embryos differed in alkaline phosphatase activity from those of the resting zone.

Both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ regulate membrane enzymes in a non-genomic manner as well. ^{45}Ca flux (Langston et al., 1990), fatty acid metabolism (Schwartz *et al.*, 1990; Swain *et al.*, 1992), and membrane fluidity (Swain *et al.*, 1993) are regulated rapidly and directly by vitamin D metabolites. Nongenomic effects of 1,25-$(OH)_2D_3$ have been

reported in other systems as well. 1,25-$(OH)_2D_3$ has been shown to stimulate the hydrolysis of membrane phosphoinositides in enterocytes (Lieberherr *et al.*, 1989; Wali *et al.*, 1990), keratinocytes (McLaughlin *et al.*, 1987; Tang *et al.*, 1987), osteoblasts (Oshima *et al.*, 1987) and parathyroid cells (Bourdeau *et al.*, 1990). Breakdown of phosphoinositides generates second messengers such as inositol triphosphate (IP_3), which releases Ca^{2+} from intracellular stores and diacylglycerol (Berridge, 1987). This biochemical pathway would be consistent with the hypothesis that PKC plays a significant role in signal transduction mediated by vitamin D metabolites.

C. Transforming Growth Factor-Beta (TGF-β):

TGF-β belongs to a family of polypeptide growth factors that include a number of homologous dimeric proteins which share a high degree of homology. Other related members of the family are the activins and inhibins (which modulate the in vitro secretion of follicle-stimulating hormone), Mullerian-inhibiting substance (which inhibits the formation of Mullerian ducts in male embryos) and products of the decapentaplegic gene complex in Drosophila. The amino acid sequences of these proteins are approximately 25-35% homologous with TGF-β (Graves and Cochran, 1990). Growth factors, in general, share a number of features in common. First, they are proteins that affect cellular activity by binding to high affinity plasma membrane receptors. Secondly, they act locally in a paracrine or autocrine manner. Thirdly, they appear to be tightly regulated in normal cells. Fourthly, they are multifunctional, affecting cellular events such as mitogenesis, cell migration, and differentiation. Lastly, their activity in vivo is probably the result of the interaction of multiple growth factors.

The original isolation and characterization of TGF-β1 began in the late 1970's when sarcoma growth factor was described (DeLarco and Todaro, 1978). DeLarco and Todaro found that mouse 3T3 cells transformed by Moloney sarcoma virus were able to induce a "transformed" phenotype in non-neoplastic, rat fibroblasts. This transformed phenotype was

characterized by the loss of density-dependent growth in culture, and by the ability to grow in soft agar (anchorage independence), an attribute observed in malignant fibroblasts but not seen in the original rat fibroblasts. This transforming activity of the sarcoma growth factor was reversible when it was removed from the cells. This transformation was observed in tumor cells other than sarcomas, thus the name was changed to "transforming growth factor" (Roberts *et al.*, 1980). TGF-β was subsequently identified in bone matrix, cartilage, platelets, lymphocytes, and other tissues (Sporn *et al.*, 1986).

TGF-β is a 25 kDa molecule consisting of two polypeptides joined by disulfied bonds (Roberts *et al.*, 1983). Three distinct forms have been identified in mammals and have been designated TGF-β1, TGF-β2 and TGF-β3. TGF-β1 and TGF-β2 can form three isoforms: a homodimer of TGF-β1, a homodimer of TGF-β2, or a heterodimer TGF-β1.2 (Graves and Cochran, 1990). TGF-β1 is synthesized as a large 390 amino acid residue molecule and is processed to a 112 amino acid polypeptide. This processed dimer is linked with the remainder of the precursor molecule, which acts as a binding protein. TGF-β1 is secreted in latent form and activated when the precursor fragment is dissociated, under conditions of low pH as found in wound healing and bone remodeling environments (Graves and Cochran, 1990). In mammals, the primary amino acid sequence for TGF-β1 is highly conserved, and is identical in man, pig, cow and monkey, and is different by only one residue in mice (Graves and Cochran, 1990). TGF-β4 has recently been cloned from chickens (Graves and Cochran, 1990) and represents another member of the family.

The *in vitro* effects of TGF-β are varied. Depending on the cell type, TGF-β may stimulate or inhibit proliferation, block a particular differentiation pathway, stimulate extracellular matrix formation, and promote or prevent cell migration (Barnard *et al.*, 1990). One of the most profound effects of TGF-β is its promotion of extracellular matrix formation. This occurs as a result of increased extracellular matrix protein synthesis, and inhibition of proteinase mediated matrix degradation (Barnard *et al.*, 1990). These important influences of

TGF-β on extracellular matrix remodelling have significant implications for its role in wound and tissue repair, bone formation and remodeling, and embryogenesis (Barnard *et al.*, 1990; Bernstein *et al.*, 1991; Beck *et al*, 1991; Chen *et al.*, 1992).

Rosier *et al.*, (1989) studied the effect of TGF-β on chick growth plate chondrocytes. They found that TGF-β significantly increased DNA synthesis, while collagen synthesis and cellular and matrix vesicle alkaline phosphatase activity were inhibited. TGF-β showed increasing mitogenicity with increasing cellular maturation in these cultures. TGF-β production by chondrocytes was also identified and suggests a potential autocrine role for this growth factor in regulating chondrocyte proliferation and matrix synthesis. Bonewald *et al.*, (1992) added both TGF-β and 1,25-$(OH)_2D_3$ to MG-63 human osteosarcoma cells and found a synergistic increase in alkaline phosphatase specific activity and collagen type I. This combination of factors was found to induce differentiation of the osteoblast to produce a "mature" extracellular matrix, but was unable to bring about mineralization. Pfeilschifter *et al.*, (1987) using ROS 17/2.8 osteosarcoma cells, found that TGF-β increased alkaline phosphatase activity and collagen synthesis per cell. This increase in activity was seen after 24 hours and could be completely inhibited with actinomycin D or cycloheximinde. Bonewald *et al.*, (1990) found that TGF-β was able to regulate both alkaline phosphatase and phospholipase A_2 in both plasma membranes and matrix vesicles produced by this cell line. They concluded that the ability of TGF-β to regulate enzymes associated with calcification, suggests that mineralization, in some way, must be influenced by TGF-β.

In vivo evidence of increased extracellular matrix synthesis was found in studies where TGF-β was injected subcutaneously into neonatal mice resulting in the development of granulation tissue at the site of injection (Roberts *et al.*, 1986). The application of TGF-β to skin wounds in rats resulted in an accelerated rate of healing as measured by wound tensile strength (Mustoe *et al.*, 1987).

TGF-β can also induce osteogenesis and chondrogenesis in the rat femur. Joyce *et al.*, (1990) injected TGF-β1 or 2 into the subperiosteal region of newborn rat femurs resulting in intramembranous bone formation and chondrogenesis. They concluded that mesenchymal precursor cells in the periosteum can be stimulated by TGF-β to proliferate and differentiate in a manner similar to that occurring in embryologic bone formation and fracture healing.

The presence of TGF-β in bone matrix and its ability to differentially regulate the enzyme activities of both osteoblast-like cells and chondrocytes in culture implicate this growth factor in being an important regulator of bone and cartilage metabolism.

D. Specific Research Objectives:

The goal of this thesis is to better understand the biochemical mechanisms used by chondrocytes in response to treatment with TGF-β and vitamin D_3 metabolites to effect changes in metabolism. This thesis will examine the role of PKC, tyrosine kinases, and phospholipase C mediating the effect of TGFβ and vitamin D_3 on rat costochondral chondrocytes in culture (Boyan *et al.*, 1988).

The following objectives outline the course of study:

1. Conduct dose-dependence and time course experiments to determine the optimal time and concentration of vitamin D_3 metabolite and TGF-β for both growth zone and resting zone chondrocytes.

2. Determine the effect of pretreatment with the phospholipase C inhibitor, U73122, or the tyrosine kinase inhibitor, genistein, on TGF-β or vitamin D metabolite-mediated PKC activity.

3. Perform Western Blot analysis to identify PKC isoforms in chondrocytes treated with either vitamin D_3 metabolites or TGF-β.

4. Determine the effect of actinomycin D and cycloheximide on the activation of PKC by TGF-β or vitamin D_3 metabolites in growth zone and resting zone chondrocytes.

5. Determine if there is a translocational shift of PKC from the cytosol to the plasma membrane as a result of treating growth zone and resting zone cells with TGF-β or vitamin D_3 metabolites.

II. MATERIALS AND METHODS:

A. Materials:

Trypsin, Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were purchased from Flow Laboratories (McLean, VA). Vitamin D_3 metabolites were gifts of Dr. Milan Uskokovic of Hoffman-LaRoche (Nutley, NJ). Recombinant human transforming growth factor β-1 (rhTGF-β-1) was purchased from R & D Systems, Inc. (Minneapolis, MN). Protein kinase C assay reagents and protein kinase C inhibitor peptide (amino acids 19-36 of pseudosubstrate region of the protein kinase C molecule) were obtained from GIBCO-BRL (Gaithersburg, MD). Pan-specific protein kinase C antibody, MC5, was obtained from Amersham Corp. (Arlington Heights, IL). Anti-protein kinase C alpha and beta isoform-specific antibodies were obtained from GIBCO-BRL (Gaithersburg, MD); anti-delta and anti-epsilon-specific isoform antibodies were a gift of Dr. Shigeo Ohno (Department of Molecular Biology, Yokohama City University Medical School, Yokohama, Japan). The bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Co. (Rockford, Il). Genistein, actinomycin D, cycloheximide, and rat brain PKC (E.C.2.7.1.37) were purchased from Calbiochem (San Diego, CA). U73122 was a gift from Dr. John Bleasdale, The Upjohn Co. (Kalamazoo, MI).

B. Chondrocyte Cultures:

The culture system has been described previously by Boyan *et al.*, (1988). Ribcages were removed from 125 g Sprague-Dawley rats and placed in Dulbecco's modified Eagle's medium (DMEM). The resting zone and adjacent growth zone cartilage were dissected and the intervening tissue removed to limit cross-contamination of cell zones, then sliced and incubated overnight in DMEM containing antibiotics at 37° C with 5% CO_2 in air and 100% humidity. The DMEM was replaced by two 20-minute washes in Hanks' Balanced Salt Solution (HBSS), followed by sequential incubations in 1% trypsin for 1 hour and 0.02%

collagenase (GIBCO Type II) for 3 hours (Suzuki *et al.*, 1981). After enzymatic digestion, cells were seperated from debris by filtration, collected by centrifugation at 500 x *g* for ten minutes, resuspended in DMEM, and plated at a density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth zone cells as described by Rifas, *et al.* (1982). Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-fungizone, and 50 μg/ml ascorbic acid (vitamin C) in an atmosphere of 5% CO_2 in air at 37° C and 100% humidity. The media were changed at 24 hours and then at 72-hour intervals thereafter. At confluence (7-10 days), cells were subcultured using the same plating densities and technique as those described above and allowed to return to confluence. Third passage confluent cultures were subpassaged into either 24-well microtiter plates or T-25 flasks and grown to confluence for the experiments. In all experiments cells were subcultured in this manner, since previous studies have demonstrated a retention of differential phenotypic markers (Boyan *et al.*, 1988).

C. Experimental Protocols:

For each experiment, confluent cultures of fourth passage chondrocytes were treated for various periods of time with control or experimental DMEM containing various concentrations of either vitamin D_3 metabolite or rhTGF-β1. To determine the optimal time course, preliminary experiments were performed. Chondrocytes were incubated with the appropriate vitamin D_3 metabolite for 0, 3, 9, 30, or 90 minutes. For subsequent experiments, growth zone cells were treated with 10^{-10} M - 10^{-7} M 1,25-$(OH)_2D_3$ or 10^{-9} M - 10^{-7} M 24,25-$(OH)_2D_3$ for up to 24 hours. Resting zone cells were treated with 10^{-9} M - 10^{-7} M 24,25-$(OH)_2D_3$ or 10^{-9} M - 10^{-7} M 1,25-$(OH)_2D_3$ for up to 24 hours. Chondrocytes treated with rhTGF-β1 were incubated for 0, 1.5, 4.5, 6, 9, 12, 24, 48, or 72 hours. For subsequent experiments with rhTGF-β1, growth zone and resting zone cells were treated with 0.05-5 ng/ml rhTGF-β1 for up to 72 hours. Initial time-course and dose-response experiments included rhTGF-β1 concentrations as high as 5 ng/ml, while subsequent experiments used

0.11 ng/ml and 0.22 ng/ml doses, which produced a sufficient dose-response effect. After various lengths of exposure to vitamin D_3 metabolite or growth factor, the cells were washed with phosphate buffered saline (PBS), loosened from the wells with a sterile cell scraper, and lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1mM phenylmethylsulfonylfluoride and 1% NP-40) for 30 minutes on ice. The lysates were used for assay of PKC activity.

D. Protein Kinase C:

1. Assay. Chondrocyte lysates were incubated for 20 minutes with a lipid preparation (7:1, v/v) containing phorbol-12-myristate-13-acetate, phosphatidlyserine and Triton X-100 mixed micelles, which provides the necessary cofactors and conditions for optimal activity (Bell *et al.*, 1986). To this mixture, a high affinity myelin basic protein peptide (MBP, 8μM) and ^{32}P-ATP (25 μCi/ml) were added to a final assay volume of 50 μl. Following a 10-minute incubation at 30^{o} C, samples were spotted onto phosphocellulose discs and washed twice with 1% phosphoric acid and once with distilled water to remove unincorporated label prior to placement in the scintillation counter. The phosphocellulose disc strongly binds the MBP peptide substrate which becomes ^{32}P-labeled during the protein kinase C reaction and unincorporated ^{32}P-ATP is washed free of the discs during the phosphoric acid wash step.

2. Inhibitor Peptide. To verify that the kinase activity was due to authentic PKC, and not due to other protein kinases, a specific inhibitor peptide corresponding to amino acid residues 19-36 of the protein kinase C pseudosubstrate region, was added to the protein kinase C reaction tubes at a final concentration of 3 μM.

3. Western Blot. To verify that protein kinase C was present in the chondrocytes, the following protocol was used. T-25 flasks of confluent fourth passage resting zone chondrocytes were lysed in 2 ml of solubilization buffer for 30 minutes on ice and centrifuged at 15,000 x *g* for 1 minute to remove debris. The lysates were then incubated for 1 hour with

20 µl of a pan-specific anti-protein kinase C IgG (Amersham Corp., Arlington Heights, IL), followed by either a 4-hour or 16-hour incubation with 20 µl of protein A-agarose (Oncogene Science, Inc., Uniondale, NY). The immunoprecipitated pellet was washed twice with solubilization buffer, twice with ultrapure deionized water, and then boiled for 4 minutes in loading buffer. Prior to electrophoresis, the sample was centrifuged for 30 seconds and the supernatant applied to a 7.5% SDS-polyacrylamide gel (8-12 hours at 10-20 mA). Authentic purified rat brain protein kinase C was run alongside the experimental samples to facilitate determination of the chondrocyte protein kinase C band(s). The gels were transferred to Immobilon-P membranes using a semi-dry electroblotter (American Bionetics, Hayward, CA) (2.5 mA/cm for 1.5 hours). The membranes were then blocked overnight in 5% nonfat dry milk and probed with an anti-protein kinase C primary antibody for one hour. This was followed by horseradish peroxidase-conjugate anti-IgG secondary antibody for one hour. Each antibody was diluted 1:500 in PBS-0.1% Tween-20 (PBST) and the membranes were washed three times with PBST before and after addition of the secondary antibody. Visualization of the protein kinase C bands was achieved using a luminol-based enhanced chemiluminescence (ECL) kit (Amersham Corp., Arlington Heights, IL) for one minute. The membrane was then exposed to Kodak XAR-5 film and the film developed.

4. Immunoprecipitation. To establish which of the protein kinase C isoforms is present in the chondrocytes, the following protocol was used. T-25 flasks of confluent fourth passage resting zone chondrocytes, pretreated 24 hours with control DMEM plus 10% FBS with or without rhTGF-β1, were lysed in 3ml of solubilization buffer for one hour on ice. Lysates were then spun for one minute at 15,000 x g to remove debris and aliquots removed for protein analysis before dividing samples into six equivalent volumes. These samples were then incubated with 10µl of PBS or 10µl of 1:2000 dilutions of anti-protein kinase C isoform specific antibodies (anti-α, β, δ, or ε) or nonspecific goat anti-mouse immunoglobulin on ice for one hour. Each sample then received 10µl of protein A-agarose, followed by a four-hour

incubation at 4°C with agitation to precipitate the protein kinase C-antibody complexes. Protein kinase C activity remaining in the cleared lysates was assayed as described above.

E. Translocation Experiments:

For PKC translocation experiments, fourth passage growth zone or resting zone cells were cultured and treated with control DMEM or DMEM containing 0.11 or 0.22ng/ml rhTGF-β1 for 12 hours. In addition, growth zone chondrocytes were treated with control media or 10^{-9}-10^{-8}M 1,25-$(OH)_2D_3$ for nine minutes, and resting zone chondrocytes were treated with control media or 10^{-8} to 10^{-7}M 24,25-$(OH)_2D_3$ for 90 minutes. Membrane and cytosol fractions were isolated using a modification of a procedure used to investigate protein kinase C translocation in osteosarcoma cells (Abou-Samra *et al.*, 1989). The cells were pelleted by centrifugation at 100 x *g* for ten minutes, resuspended in ice-cold homogenization buffer containing 20mM Tris-HCl, pH 7.4, 2mM EDTA, 0.5mM phenylmethylsulfonylfluoride plus 1% NP-40, and homogenized on ice in a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 100,000 x *g* for 45 minutes, and the pellet containing the membranes was resuspended in homogenization buffer plus 1% NP-40. Protein concentration and protein kinase C activity were assayed in the cytosol and membrane fractions from control and either vitamin D_3 or rhTGF-β1-treated chondrocyte cultures.

F. Tyrosine Kinase and Phospholipase C:

In order to determine whether tyrosine kinase or phospholipase C was involved in the mechanism of protein kinase C activation, growth zone or resting zone chondrocyte cultures were pretreated with 0.1 - 10 μM of either the tyrosine kinase inhibitor, genistein, or the phospholipase C inhibitor, U73122. Cultures were pretreated with control media or inhibitors for 2 hours at which time the media were changed to control or experimental media containing vitamin D_3 metabolite or rhTGF-β1. Protein concentration and protein kinase C activity were measured. Cell viability was determined using trypan blue dye exclusion for chondrocyte cultures exposed to media containing 10 μM genistein or 10 μM U73122 for 72 hours.

G. Transcription and Translation:

To examine the involvement of genomic mechanisms in protein kinase C activation, the chondrocyte cultures were treated with vitamin D_3 metabolites or rhTGF-β1 in the presence of the transcription inhibitor, actinomycin D, or the translation inhibitor, cycloheximide. Growth zone chondrocytes were incubated with control or 10^{-9} - 10^{-8} M 1,25-$(OH)_2D_3$ and 0.01 or 0.1 mM actinomycin D or cycloheximide for 9 minutes. Resting zone chondrocytes were incubated with control or 10^{-8} M - 10^{-7} M 24,25-$(OH)_2D_3$ and 0.01 or 0.1 mM actinomycin D or cycloheximide for one hour. Both growth zone and resting zone chondrocytes were incubated with control, 0.11, 0.22, or 10 ng/ml rhTGF-β1 and 0.01 or 0.1 mM actinomycin D or cycloheximide for 12 hours. Following treatment with the inhibitors, the cells were washed with PBS and assayed for protein kinase C activity.

H. Protein Determination:

The protein content of each experimental sample was determined using the Macro BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). This was accomplished so that enzyme specific activities could be calculated for each sample. This assay system is highly sensitive for the determination of protein concentration in dilute solution. Bicinchoninic acid (BCA), in the form of a water-soluble salt, is a sensitive reagent for copper. The Macro BCA Protein Assay Reagent combines the biuret reaction (Protein reacting with Cu^{2+}) with BCA. A purple product is formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{1+}), and exhibits a strong absorbance at 562 nm. This absorbance was measured spectrophotometrically with an automatic densitometer (BioRad, Inc., Richmond, CA).

I. Statistical Analysis:

Data on the regulation of protein kinase C activity by vitamin D_3 metabolites or rhTGF-β 1 are expressed as the mean pMol phosphate transferred/μg protein/minute ± standard error of the mean (SEM) of six cultures. Observations were validated by independent experimental

replicates (minimum of two). Significance between treatment and controls was determined by the Bonferroni's *t*-test using $P<0.05$ confidence limits.

III. RESULTS

A. Vitamin D Metabolites:

1. Time-Course. When growth zone chondrocytes were incubated from 0-90 minutes with 10^{-10} - 10^{-8} M 1,25-$(OH)_2D_3$, there was a stimulation of PKC activity that occurred as early as 3 minutes after addition of hormone (Table 1) and was significant at 10^{-8} M. Subsequent experiments, over longer periods of time, showed that peak stimulation occurred at 9 minutes and was sustained for 90 minutes (Figure 1A). In another experiment, this profile was reproducible, and no subsequent increase in activity was observed at 6, 12, or 24 hours following 1,25-$(OH)_2D_3$ exposure. 24,25-$(OH)_2D_3$ did not activate PKC in growth zone chondrocyte cultures at any of the times and concentrations tested (Figure 1B), nor at 6, 12, or 24 hours.

1,25-$(OH)_2D_3$ did not activate PKC in resting zone cell cultures at any dose or time examined (Figure 2A). Resting zone cell cultures did exhibit increased PKC activation in response to stimulation by 24,25-$(OH)_2D_3$. A significant increase in activity began between 9 and 30 minutes after addition of hormone and reached a maximum at 90 minutes. High levels of activity were sustained for an additional 180 minutes after reaching their peak levels. The effect of hormone was still significant at 4.5 hours after addition in cultures exposed to 10^{-8} - 10^{-7} M 24,25-$(OH)_2D_3$ (Figure 2B).

2. Specificity to PKC. The protein kinase activity regulated by vitamin D_3 metabolites in chondrocytes was confirmed to be PKC by addition of a specific competitive inhibitor peptide (Table 2). The PKC inhibitor at a final concentration of 3 μM reduced 1,25-$(OH)_2D_3$ -stimulated PKC activity in growth zone cell lysates by 95.6%. 24,25-$(OH)_2D_3$-stimulated activity in resting zone cell lysates was reduced by 85.9%.

3. Western Blot. When Western blots were probed using an anti-rat PKC antibody which recognizes all isoforms of the enzyme (Figure 3), the major immunoreactive protein

Table 1. **1,25-$(OH)_2D_3$-DEPENDENT EFFECT ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE CHONDROCYTES.**

1,25-$(OH)_2D_3$	**pMol PO_4/ug Protein/ Minute**
0	0.129 ± 0.018
10^{-10}M	0.081 ± 0.009
10^{-9}M	0.124 ± 0.023
10^{-8}M	0.781 ± 0.024 *

At confluence, growth zone chondrocytes were incubated with control medium (vehicle only) or with medium containing 1,25-$(OH)_2D_3$ for 3 minutes. Values are mean $\pm$ SEM for 6 cultures. Data are from a single experiment and were validated in a replicated experiment. *$p < 0.05$, treated vs. control.

Figure 1A. PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE CHONDROCYTES AFTER TREATMENT WITH 1,25-$(OH)_2D_3$ FOR VARIOUS PERIODS OF TIME. Confluent, fourth passage growth zone chondrocytes were treated for various periods of time with DMEM + 10% FBS and various concentrations of 1,25-$(OH)_2D_3$. Each point represents the mean $\pm$ SEM of protein kinase C specific activity for 6 cultures. Data are from one of two replicate experiments. *$p<0.05$, treatment vs control.

Growth Zone Chondrocytes

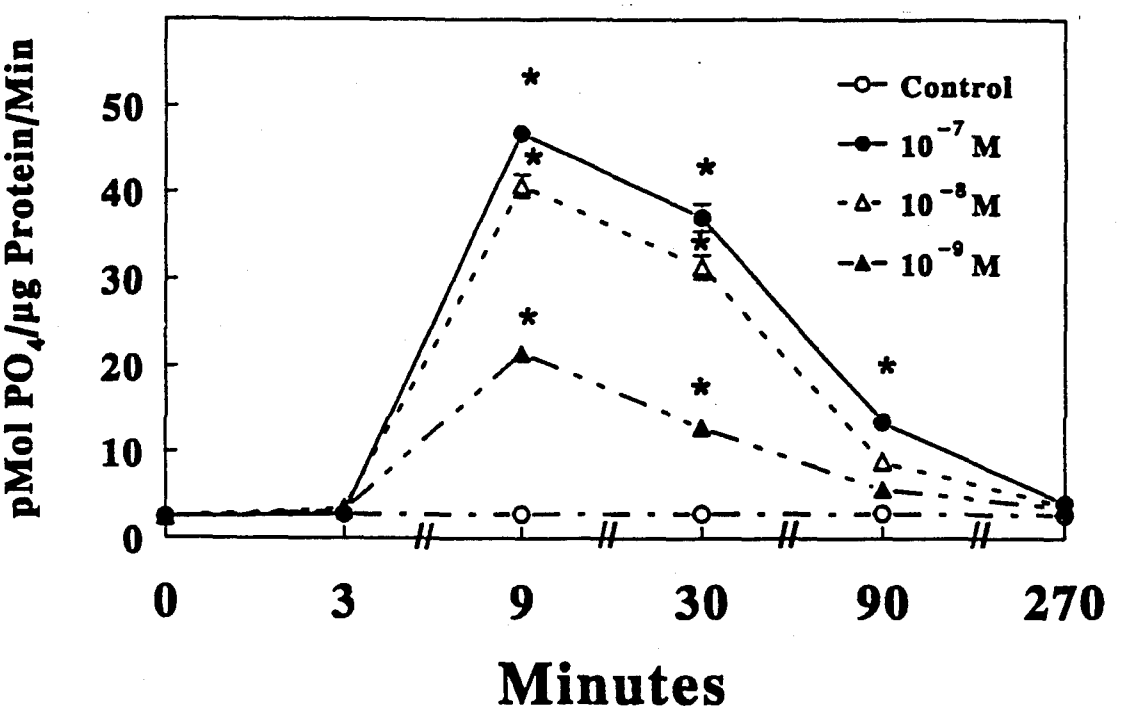


Figure 1B. PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE CHONDROCYTES AFTER TREATMENT WITH 24,25-$(OH)_2D_3$ FOR VARIOUS PERIODS OF TIME. Confluent, fourth passage growth zone chondrocytes were treated for various periods of time with DMEM + 10% FBS and various concentrations of 24,25-$(OH)_2D_3$. Each point represents the mean $\pm$ SEM of protein kinase C specific activity for 6 cultures. Data are from one of two replicate experiments. *$p<0.05$, treatment vs control.

Growth Zone Chondrocytes

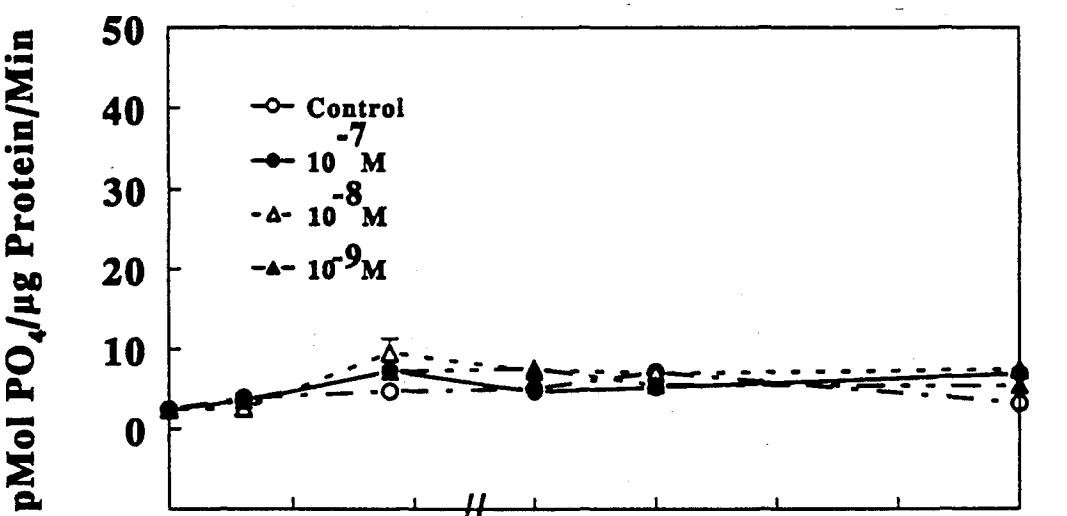


Figure 2A. PROTEIN KINASE C SPECIFIC ACTIVITY OF RESTING ZONE CHONDROCYTES AFTER TREATMENT WITH 1,25-$(OH)_2D_3$ FOR VARIOUS PERIODS OF TIME. Confluent, fourth passage resting zone chondrocytes were treated for various periods of time with DMEM + 10% FBS and various concentrations of 1,25-$(OH)_2D_3$. Each point represents the mean ± SEM of protein kinase C specific activity for 6 cultures. Data are from one of two replicate experiments. *$p<0.05$, treatment vs control.

Resting Zone Chondrocytes

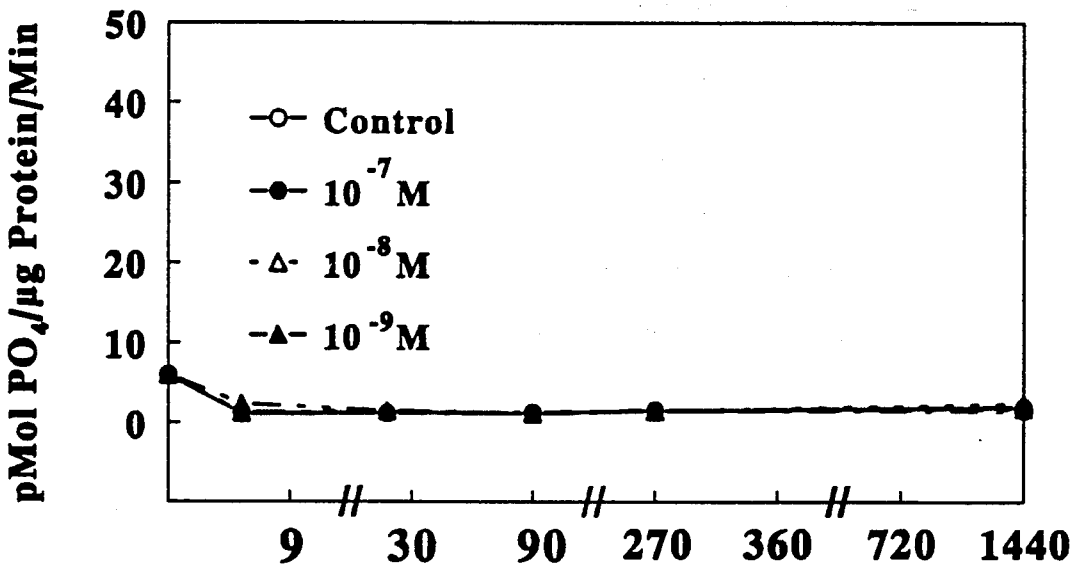


Figure 2B. PROTEIN KINASE C SPECIFIC ACTIVITY OF RESTING ZONE CHONDROCYTES AFTER TREATMENT WITH 24,25-$(OH)_2D_3$ FOR VARIOUS PERIODS OF TIME. Confluent, fourth passage resting zone chondrocytes were treated for various periods of time with DMEM + 10% FBS and various concentrations of 24,25-$(OH)_2D_3$. Each point represents the mean ± SEM of protein kinase C specific activity for 6 cultures. Data are from one of two replicate experiments. *$p<0.05$, treatment vs control.

Resting Zone Chondrocytes

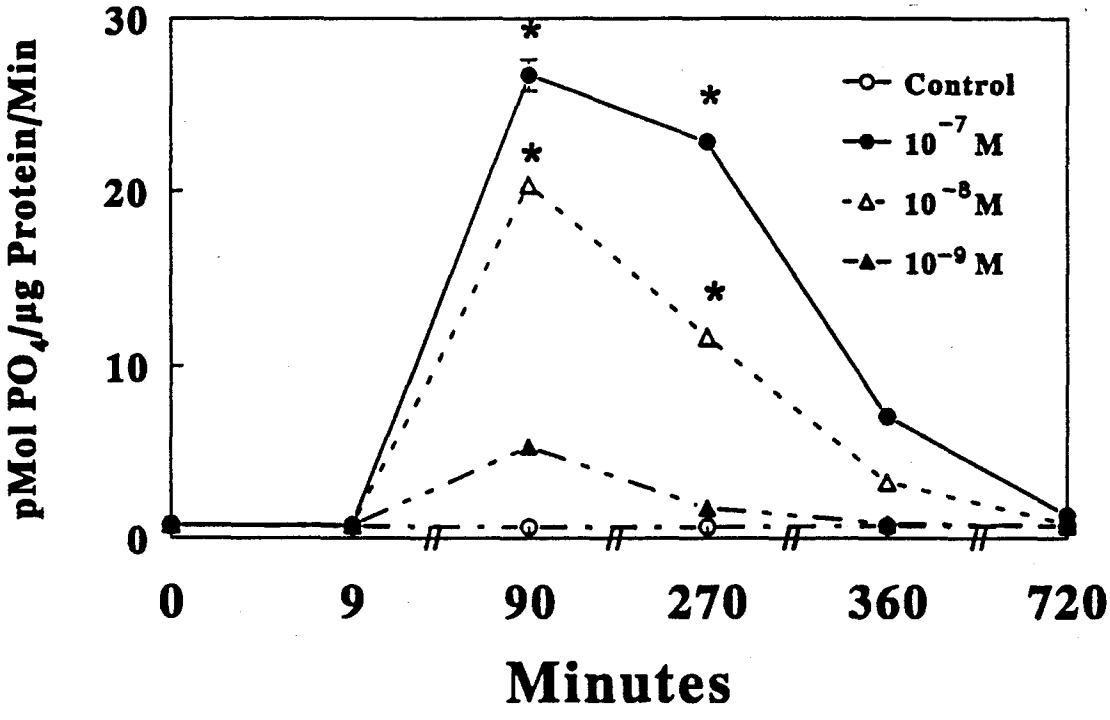
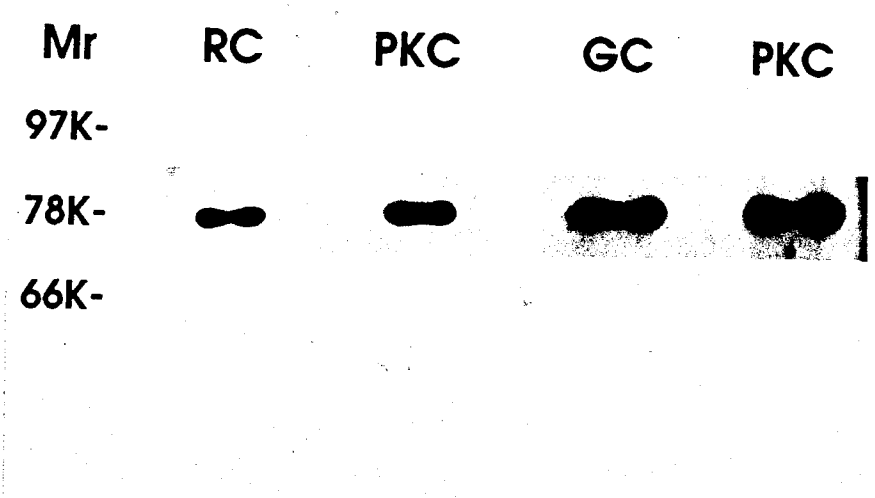


Table 2. EFFECT OF PROTEIN KINASE C INHIBITOR PEPTIDE ON PROTEIN KINASE C SPECIFIC ACTIVITY OF GROWTH ZONE (GC) AND RESTING ZONE (RC) CHONDROCYTES.

	Protein Kinase C Specific Activity pMol PO_4/μg Protein/Minute	
Treatment	**GC**	**RC**
Control	0.65 ±0.01	0.87 ± 0.01
Control + inhibitor (3 μM)	0.83 ±0.01	0.83 ± 0.01
1,25-$(OH)_2D_3$ (10^{-8}M)	8.92 ± 0.01	N.D
1,25-$(OH)_2D_3$ (10^{-8}M) + inhibitor (3 μM)	0.40 ± 0.01*	N.D
24,25-$(OH)_2D_3$ (10^{-8}M)	N.D.	10.89 ± 0.02
24,25-$(OH)_2D_3$ (10^{-8}M) + inhibitor (3 μM)	N.D.	1.53 ± 0.02*

Values represent the mean ± SEM of six cultures. Data are from a single experiment. The observations were validated in 2 replicate experiments. *$p < 0.05$, hormone + 3 uM inhibitor vs. hormone alone. N.D., not done.

Figure 3. WESTERN BLOT ANALYSIS OF PROTEIN KINASE C (PKC) PROTEIN IN RAT COSTOCHONDRAL CHONDROCYTE CELL LYSATES. Fourth passage resting zone (left panel) and growth zone (right panel) chondrocytes were cultured to confluence in T-25 flasks, lysed in solubilization buffer, immunoprecipitated with pan-specific anti-rat protein kinase C antibody, separated on a 7.5% SDS-PAGE, transferred to Immobilon-P (1 hour at 320 mA), probed with anti-protein kinase C IgG and visualized with a luminol-based detection system (ECL, Amersham Corp.). Migration of the major chondrocyte protein kinase C (first band) was identical to purified rat brain protein kinase C standard (second band). Molecular weight region 97-66 kD is shown.


Mr
RC
PKC
GC
PKC
97K-
78K-
66K-

produced by resting zone and growth zone cells had an apparent molecular weight of 78 kDa. When longer exposure times were used, numerous additional bands were detected throughout the Western blot. These were presumed to be due to aggregation, degradation, or nonspecific binding, since they did not co-migrate with authentic PKC.

4. Translocation Experiments. Upon stimulation with vitamin D_3 metabolites, PKC appears to translocate from the cytosol to the membrane (Table 3). After treatment of growth zone chondrocytes with 10^{-8} M 1,25-$(OH)_2D_3$ for 9 minutes, the amount of PKC specific activity in the cytosol increased 4.5 fold, whereas the enzyme specific activity in the membrane increased 31-fold. Similarly, after resting zone chondrocytes were exposed to 24,25-$(OH)_2D_3$ for 90 minutes, PKC specific activity in the cytosol increased 6.8-fold, while specific activity in the membranes increased 23.7-fold.

5. Tyrosine Kinase and Phospholipase C. Pretreatment with U73122 (phospholipase C inhibitor) had no effect on basal PKC specific activity levels in growth zone (Figure 4A) or resting zone (Figure 4B) cell cultures. U73122 reduced 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone cell cultures in a dose-dependent manner (Figure 4A). The inhibitory effect of U73122 was significant at concentrations of 0.1, 1, and 10 μM, reducing PKC specific activity 23%, 43%, and 78%, respectively. The phospholipase C inhibitor had no effect on 24,25-$(OH)_2D_3$-stimulated PKC specific activity in resting zone cell cultures at any concentration or time point examined (Figure 4B). When treated with the highest concentration of U73122 (10 μM), chondrocyte cultures displayed at least 98% viability, indicating that the compound was well tolerated by the cells, and the reduction in PKC specific activity was not a result of cytotoxicity.

Unlike the inhibition observed for U73122, genistein (tyrosine kinase inhibitor) had no significant effect on vitamin D_3-stimulated PKC specific activity in either growth zone or resting zone cell cultures (data not shown). Cell viability following genistein treatment was

Table 3. PROTEIN KINASE C SPECIFIC ACTIVITY IN CHONDROCYTE CYTOSOL AND MEMBRANE FRACTIONS AFTER TREATMENT WITH VITAMIN D METABOLITES.

Treatment	Growth Zone Cells		Resting Zone Cells	
	Cytosol	Membrane	Cytosol	Membrane
Control	2.55±0.04	0.66±0.02	3.43±0.07	0.54±0.02
1,25-$(OH)_2D_3$ (10^{-8}M)	11.56±0.69	20.44±0.46	N.D.	N.D.
24,25-$(OH)_2D_3$ (10^{-8}M)	N.D.	N.D.	23.43±0.31	12.77±0.26

Values represent the mean ± SEM for protein kinase C specific activity (pMol $PO_4/\mu g$ protein/min). Data are from a single experiment with six determinations for each condition. N.D., not done.

Figure 4A. EFFECT OF U73122 ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES INCUBATED WITH 1,25-$(OH)_2D_3$. Confluent, fourth passage growth zone chondrocytes were treated for two hours with various concentrations of U73122 followed by fresh media ± 1,25-$(OH)_2D_3$ for 9 minutes. Each value represents the mean ± SEM for six cultures. *$p < 0.05$, treatment vs. control.

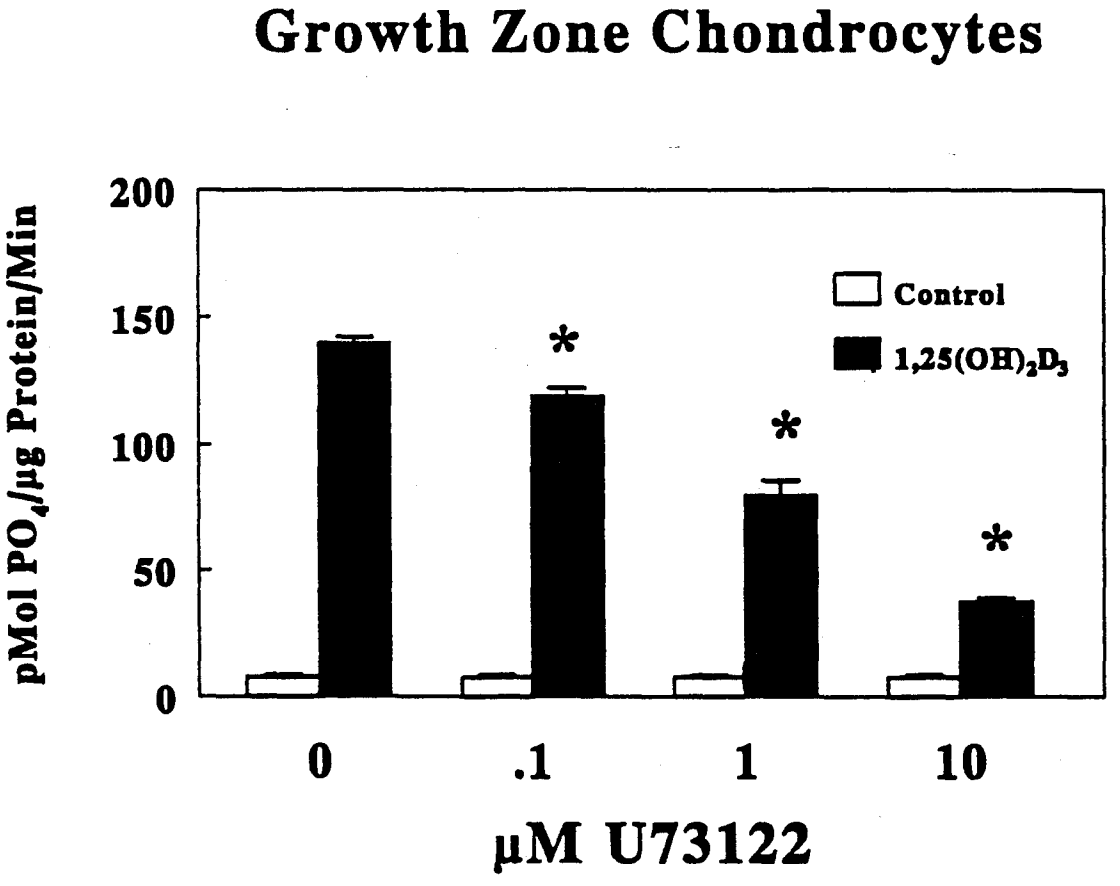
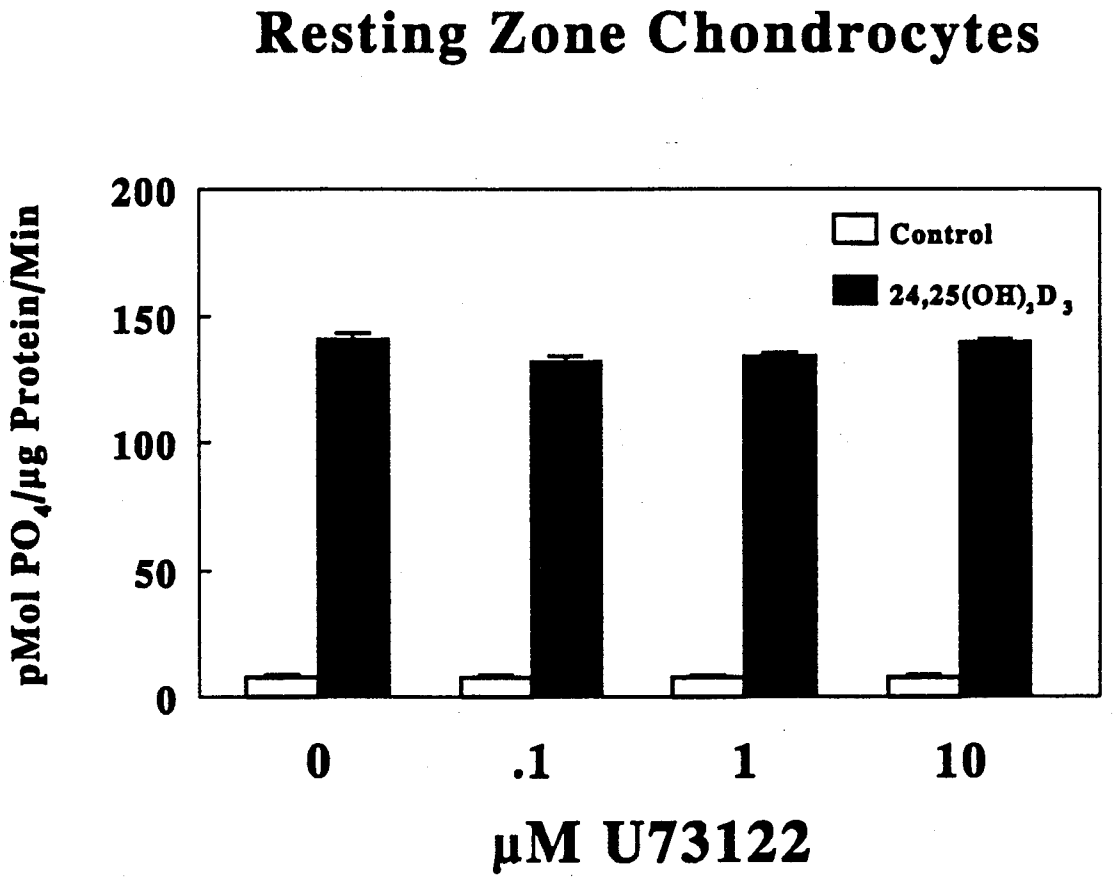

Growth Zone Chondrocytes
pMol PO4/µg Protein/Min
200
150
100
50
0
Control
1,25(OH)2D3
*
*
*
0
.1
1
10
µM U73122

Figure 4B. EFFECT OF U73122 ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES INCUBATED WITH 24,25-$(OH)_2D_3$. Confluent, fourth passage resting zone chondrocytes were treated for two hours with various concentrations of U73122 followed by fresh media $\pm$ 24,25-$(OH)_2D_3$ for 90 minutes. Each value represents the mean $\pm$ SEM for six cultures. *$p < 0.05$, treatment vs. control.


Resting Zone Chondrocytes
pMol PO4/µg Protein/Min
200
150
100
50
0
Control
24,25(OH)2D3
0
.1
1
10
µM U73122

never less than 96%, indicating that the concentrations used were not deleterious to the chondrocytes.

6. Transcription and Translation. The transcription inhibitor, actinomycin D, had no effect upon 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone cell cultures (Figure 5A). Similarly, the translation inhibitor, cycloheximide, did not alter 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone cell cultures at the concentrations tested (Figure 5B).

In contrast, actinomycin D and cycloheximide both produced dose-dependent inhibition of 24,25-$(OH)_2D_3$-stimulated PKC specific activity in resting zone chondrocytes. PKC specific activity was reduced 18.1% by 0.01 mM actinomycin D and 33.4% by 0.1 mM actinomycin D in resting zone cells treated with 10^{-7} M 24,25-$(OH)_2D_3$ (Figure 6A). Similarly, PKC specific activity was reduced 28.2% by 0.01 mM cycloheximide and 46.7% by 0.1 mM cycloheximide in resting zone cells treated with 10^{-7} M 24,25-$(OH)_2D_3$ (Figure 6B). In resting zone cells incubated with 10^{-8} M 24,25-$(OH)_2D_3$, PKC specific activity was inhibited only at the highest concentration of actinomycin D or cycloheximide.

B. rhTGF-β1:

1. Time-Course Experiments. rhTGF-β1 stimulated PKC specific activity in both growth zone and resting zone chondrocyte cultures, although the time course of response was different. In growth zone chondrocytes, stimulation of PKC specific activity by rhTGF-β1 was found at 12 and 24 hours, with significant increases observed for all three concentrations at 12 hours (Figure 7A). By 24 hours, only the highest concentration of rhTGF-β1 effected a significant increase in the specific activity of PKC. By 48 hours, the activity was reduced to control levels. For resting zone chondrocytes, a significant increase in PKC specific activity was observed at nine hours after growth factor addition in cultures treated with both 0.5 and

Figure 5A. EFFECT OF ACTINOMYCIN D ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES INCUBATED WITH 1,25-$(OH)_2D_3$. Confluent, fourth passage growth zone chondrocytes were treated with various concentrations of 1,25-$(OH)_2D_3$ for 9 minutes in the absence or presence of actinomycin D. Each value represents the mean $\pm$ SEM for six cultures. *$p < 0.05$, treatment vs. control.

Growth Zone Chondrocytes

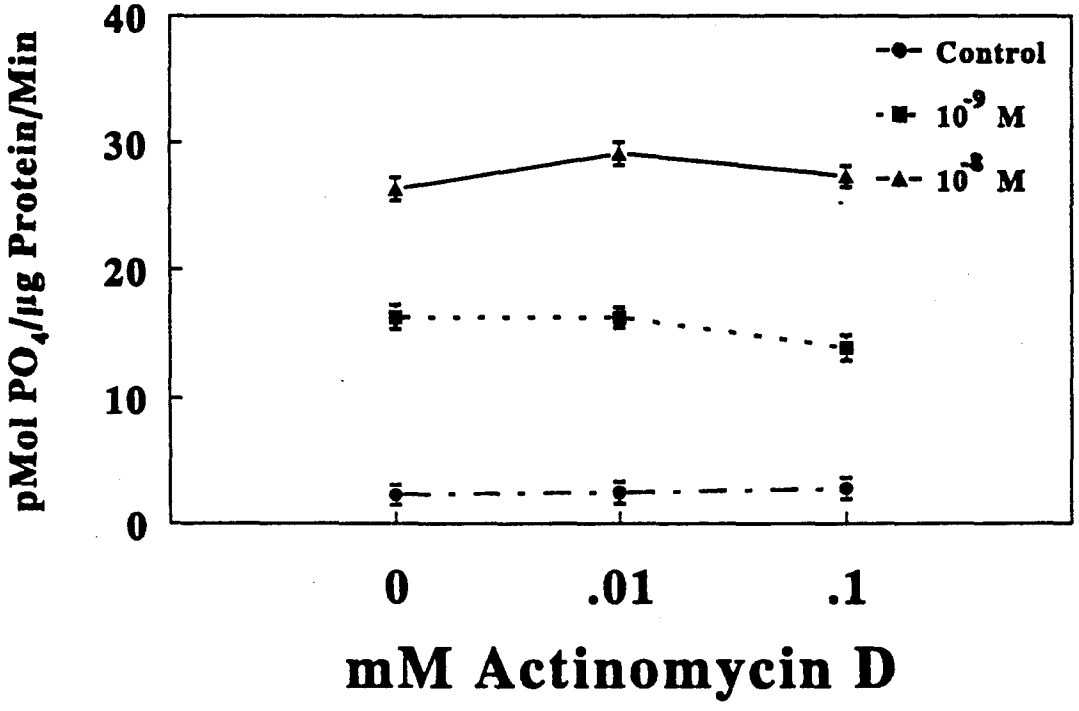


Figure 5B. EFFECT OF CYCLOHEXIMIDE ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES INCUBATED WITH 1,25-$(OH)_2D_3$. Confluent, fourth passage growth zone chondrocytes were treated with various concentrations of 1,25-$(OH)_2D_3$ for 9 minutes in the absence or presence of cycloheximide. Each value represents the mean $\pm$ SEM for six cultures. *$p < 0.05$, treatment vs. control.

Growth Zone Chondrocytes

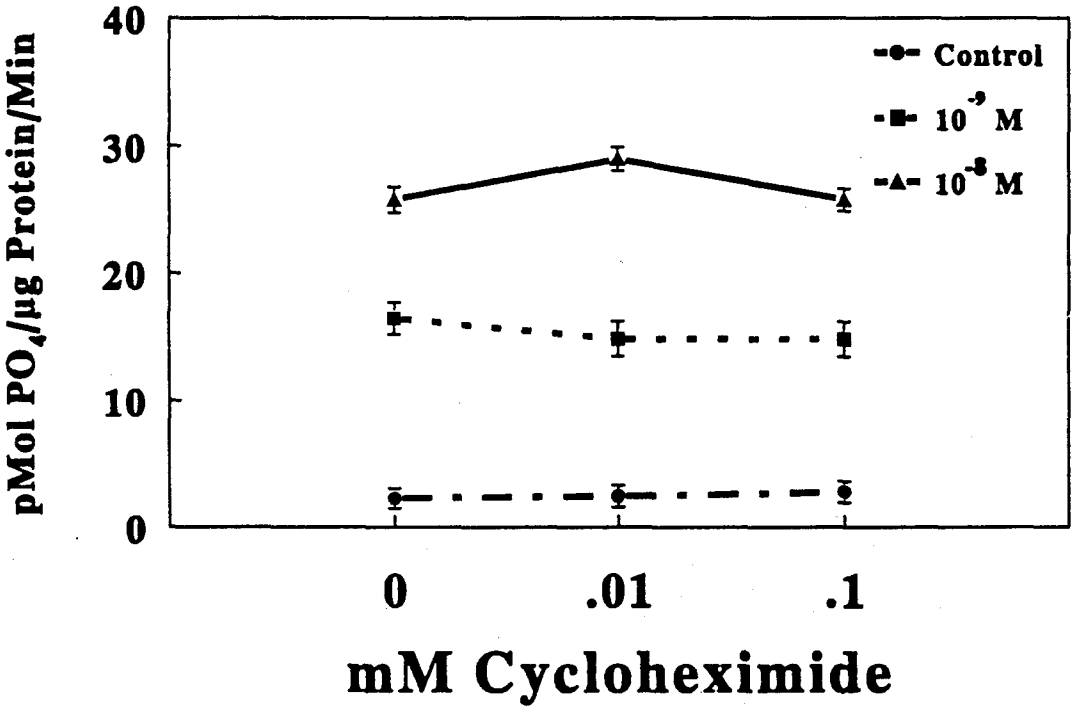


Figure 6A. EFFECT OF ACTINOMYCIN D ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES INCUBATED WITH 24,25-$(OH)_2D_3$. Confluent, fourth passage resting zone chondrocytes were treated with various concentrations of 24,25-$(OH)_2D_3$ for 90 minutes in the absence or presence of actinomycin D. Each value represents the mean $\pm$ SEM for six cultures. *$p < 0.05$, treatment vs. control.

Resting Zone Chondrocytes

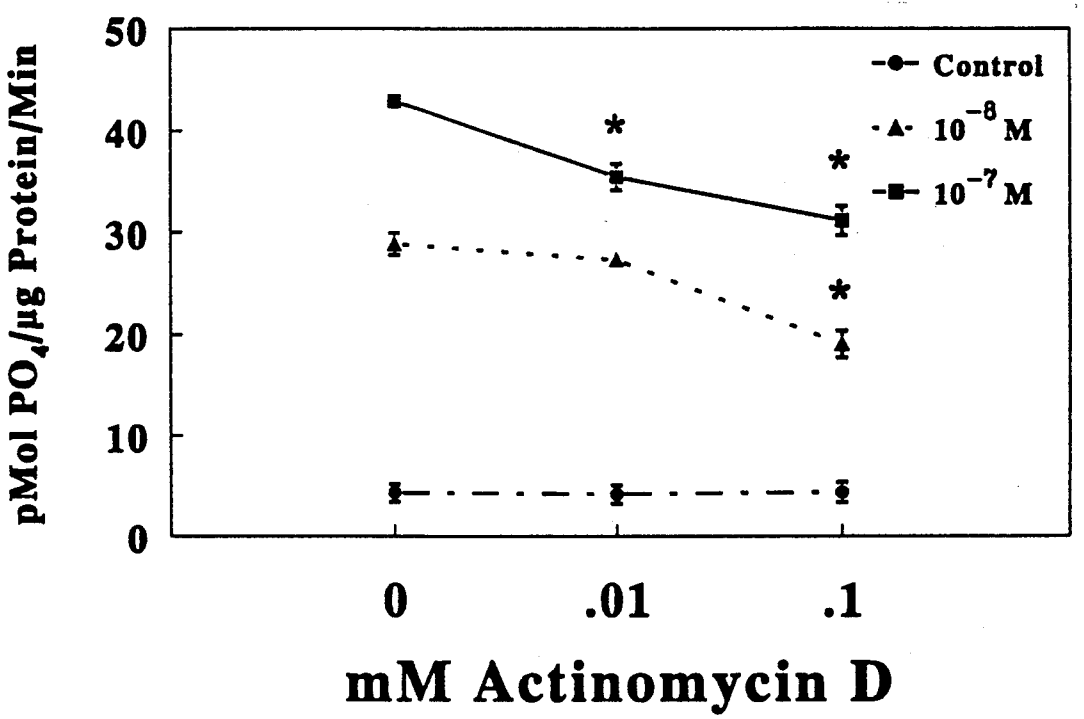


Figure 6B. EFFECT OF CYCLOHEXIMIDE ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES INCUBATED WITH 24,25-$(OH)_2D_3$. Confluent, fourth passage resting zone chondrocytes were treated with various concentrations of 24,25-$(OH)_2D_3$ for 90 minutes in the absence or presence of cycloheximide. Each value represents the mean $\pm$ SEM for six cultures. *$p < 0.05$, treatment vs. control.

Resting Zone Chondrocytes

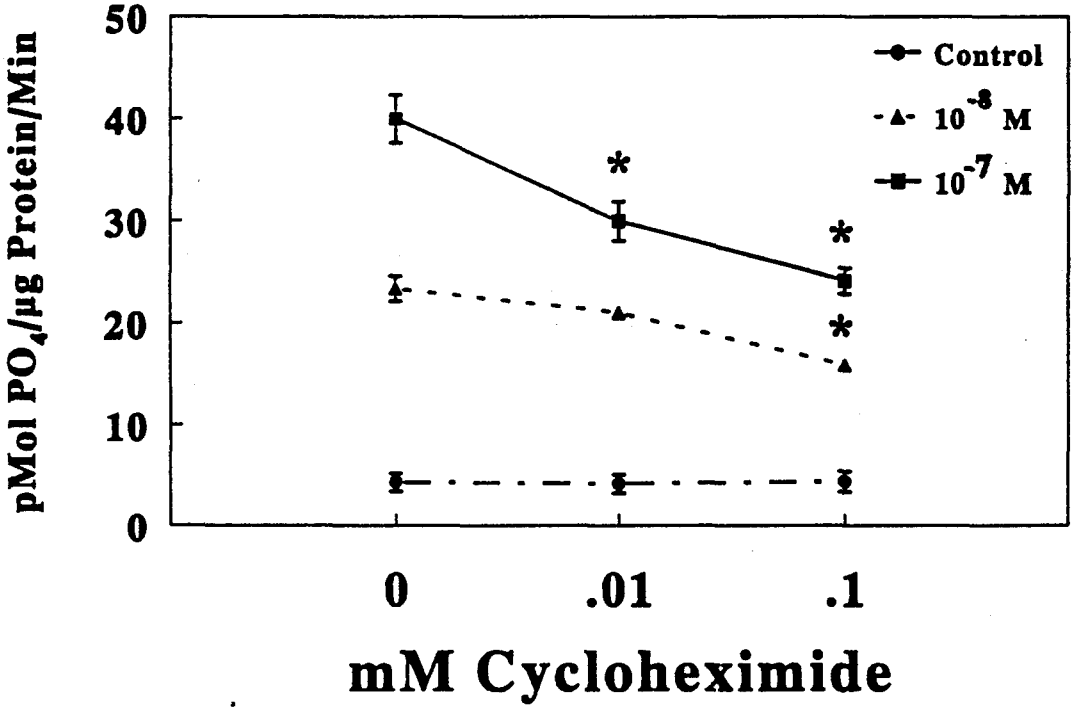
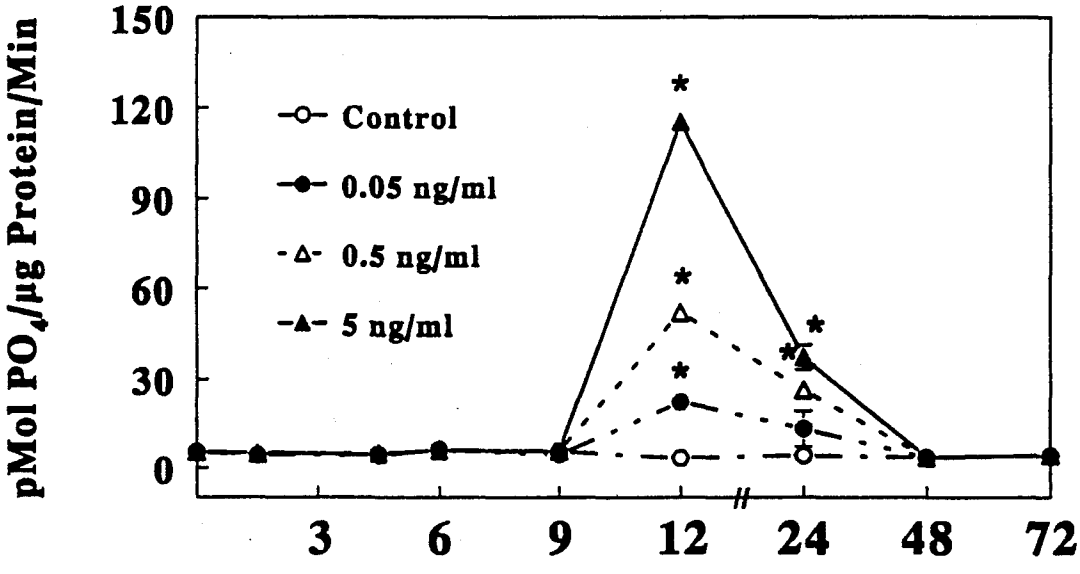


Figure 7A. STIMULATION OF PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES AFTER TREATMENT WITH rhTGF-β1. Confluent, fourth passage growth zone chondrocytes were treated for various incubation times with 0-5 ng/ml recombinant human rhTGF-β1 in DMEM + 10% FBS. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of three replicate experiments. *P<0.05, treatment vs. control.

Growth Zone Chondrocytes



5ng/ml rhTGF-β1. Peak stimulation was observed at 12 hours and continued for 24 hours before returning to control levels at 48 hours (Figure 7B). These time courses of activation were reproducible in at least three independent experiments for both growth zone and resting zone chondrocyte cultures. Occasionally, activation was observed as early as six hours following exposure to rhTGF-β1 in resting zone cells; however, peak stimulation was always seen at the 12-hour time point.

2. Specificity to PKC. We verified that kinase regulation in chondrocytes by TGF-β 1 was indeed PKC and not catalyzed by other cellular kinases. A PKC inhibitor peptide, at a final concentration of 3μM, reduced rhTGF-β1-stimulated PKC specific activity in growth zone cell lysates by 87.3%, and by 88.3% in resting zone cell lysates, with no discernible effect upon basal PKC specific activity (Table 4).

3. Western Blot. Detection of PKC protein in resting zone chondrocyte cultures by Western blot analysis using a pan-specific antibody showed a single major immunoreactive species in the molecular weight range of 77-83 kDa that comigrated with a PKC standard purified from rat brain (Figure 8). The PKC bands observed in immunoprecipitates from control and rhTGF-β1-treated resting zone chondrocyte cultures have a calculated molecular weight of 78kDa. The blot shows that, while the intensity of additional lower molecular weight bands is less in the rhTGF-β1-treated sample lane, the intensity of the PKC band is greater.

4. Immunoprecipitation. Only anti-PKC alpha reduced activity in resting zone chondrocyte lysates, while specific antibodies to the β, δ, and ε isoforms of PKC had no effect (Table 5). Treatment with non-specific IgG reduced PKC specific activity by 19.6% in control lysates and by 27.9% in lysates from rhTGF-β1-treated cells compared to lysates in the absence of non-specific IgG. Anti-β, -δ and -ε antibodies produced similar results, reducing PKC specific activity by an average of 21.9% for lysates from control cells

Figure 7B. STIMULATION OF PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES AFTER TREATMENT WITH rhTGF-β1. Confluent, fourth passage resting zone chondrocytes were treated for various incubation times with 0-5 ng/ml recombinant human rhTGF-β1 in DMEM + 10% FBS. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of five replicate experiments. *P<0.05, treatment vs. control.

Resting Zone Chondrocytes

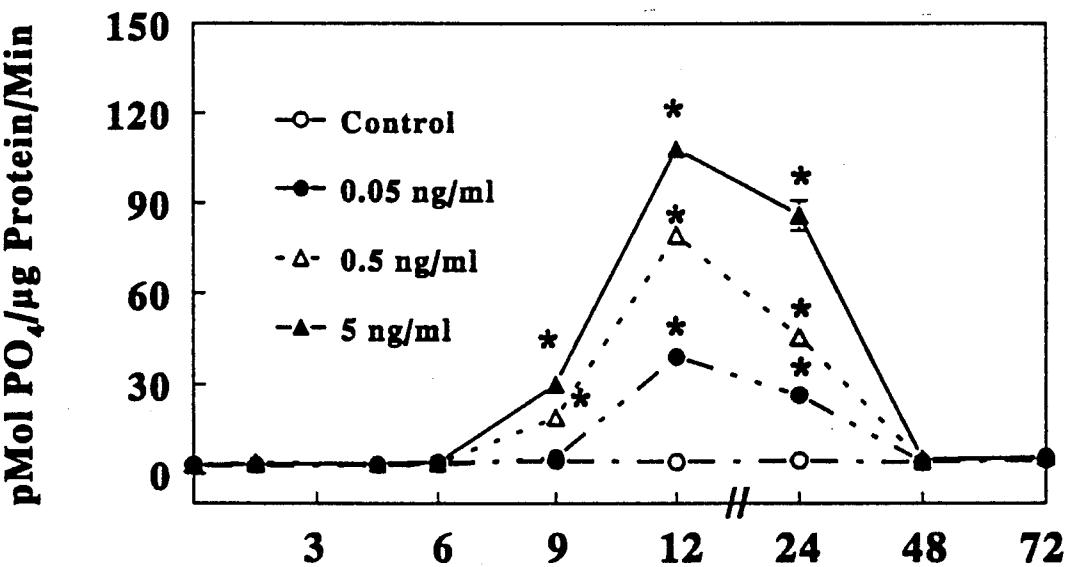


Table 4. EFFECT OF PROTEIN KINASE C INHIBITOR PEPTIDE ON rhTGF-β1-INDUCED PROTEIN KINASE C SPECIFIC ACTIVITY IN COSTOCHONDRAL CHONDROCYTES.

	Protein Kinase C Specific Activity pMol PO_4/μg Protein/ Minute	
Treatment	**GC**	**RC**
Control	6.5 ± 0.1	8.7 ± 0.1
Control + Inhibitor	8.3 ± 0.1	8.3 ± 0.1
TGFβ	99.6 ± 0.1	116.5 ± 0.3
TGFβ + Inhibitor	11.1 ± 0.1*	14.8 ± 0.3*

Protein kinase C specific activity (pMol PO_4/μg protein/min) is expressed as the mean ± SEM of six cultures treated for 12 hours with control media or 5ng/ml rhTGF-β1 (TGFβ). Inhibitor peptide was added at the time of assay at a concentration of 3μM. Data are from a single experiment and the observations were validated in two replicate experiments. GC: growth zone chondrocytes; RC: resting zone chondrocytes; *$p < 0.05$, TGFβ + inhibitor vs. TGFβ alone.

Figure 8. WESTERN BLOT ANALYSIS OF PROTEIN KINASE C PROTEIN IN RAT COSTOCHONDRAL CHONDROCYTE CELL LYSATES TREATED WITH CONTROL OR TGFβ. Fourth passage resting zone chondrocytes were cultured to confluence and treated with control DMEM + FBS (lanes 2,3) or 5 ng/ml rhTGF-β1 (lane 4,5) for 24 hours, lysed in solubilization buffer, immunoprecipitated with pan-specific protein kinase C antibody (lanes 2 and 4) or nonspecific goat anti-mouse IgG (lanes 3 and 5), separated on a 7.5% SDS-PAGE, transferred to Immobilon P (1 hr at 320 mA), probed with anti-protein kinase C IgG and visualized with a luminol-based detection system (ECL, Amersham Corp.). Migration of the major chondrocyte protein kinase C was identical to purified rat brain protein kinase C standard (lane 6). Prestained molecular weight markers shown in lane 1.

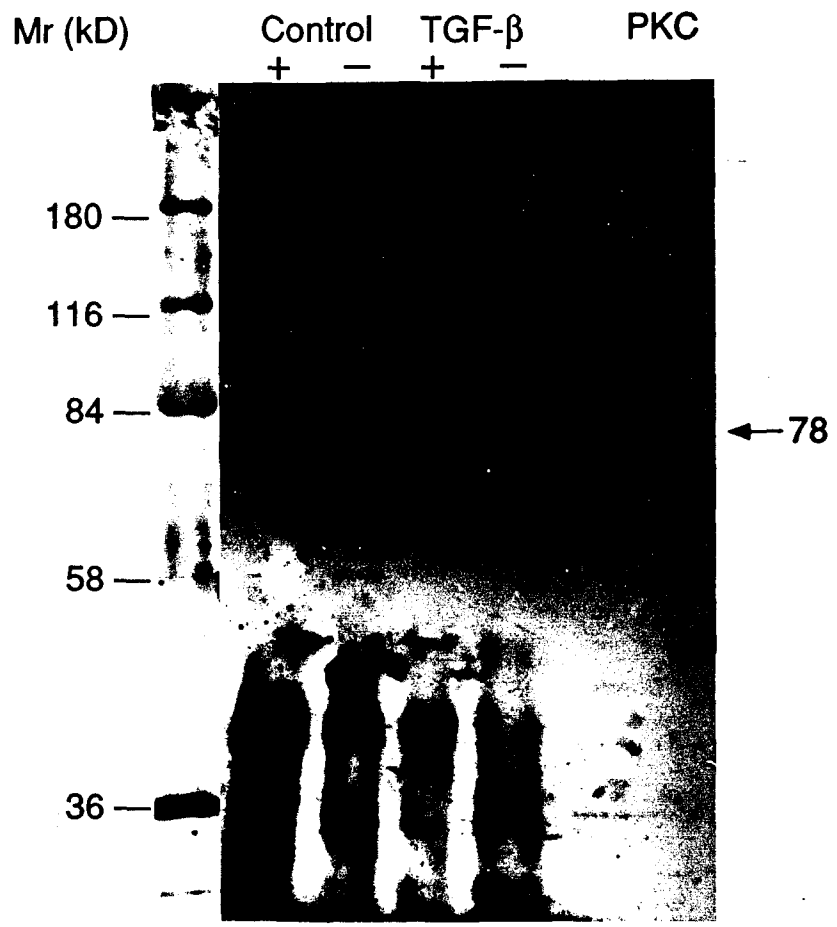

Mr (kD)
Control
TGF-β
PKC
+
−
+
−
180
116
84
58
36
78

Table 5. EFFECT OF ISOFORM-SPECIFIC ANTIBODIES ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTE CULTURES.

Antibody	Control	TGFβ
None	100	100
Non-specific IgG	80.4 ± 5.9	72.1 ± 2.8
Alpha	***34.3 ± 0.6****	***7.8 ± 0.2****
Beta	78.3 ± 2.4	66.6 ± 1.2
Delta	81.5 ± 1.4	76.3 ± 1.7
Epsilon	74.6 ± 8.3	75.2 ± 0.6

Protein kinase C specific activity is expressed as % control of activity remaining in untreated and 12-hour rhTGF-β1-treated resting zone chondrocyte lysates prior to and following treatment with anti-protein kinase C isoform-specific antibodies. Values represent the mean ± SEM for six cultures. *$P<0.05$, specific antibody vs. nonspecific IgG.

and 27.3% from TGFβ-treated cells. Anti-α antibody, however, reduced PKC specific activity by 65.7% in lysates of controls and by 92.2% for TGFβ-treated cells. PKC specific activity in the rhTGF-β1-treated chondrocytes was decreased to a greater extent than in the control cells.

5. Translocation. rhTGF-β1 does not appear to promote substantial translocation of PKC to the cell membrane at the time points and concentrations examined (Table 6). The level of PKC specific activity in membrane fractions isolated from growth zone chondrocyte cultures exposed to 0.11ng/ml rhTGF-β1 for 12 hours was 2.3-fold greater than control membranes, while those exposed to 0.22ng/ml were only 1.7-fold greater. Following a 12-hour exposure to 0.11ng/ml rhTGF-β1, there was a 3.0-fold increase in activity in resting zone cell membranes, while 0.22ng/ml only produced a 1.8-fold increase. In contrast, dose dependent increases in the cytosolic enzyme activity were observed. Growth zone cells exhibited a 1.9-fold increase when incubated with 0.11 ng/ml rhTGF-β1 and a 4.2-fold increase when incubated with 0.22 ng/ml rhTGF-β1. Resting zone cells exhibited a 3.4-fold increase when incubated with 0.11 ng/ml rhTGF-β1 and a 6.1-fold increase at 0.22 ng/ml rhTGF-β1. Unlike cells treated with rhTGF-β1, chondrocytes treated with their specific vitamin D metabolite showed translocation of PKC from cytosol to the membrane.

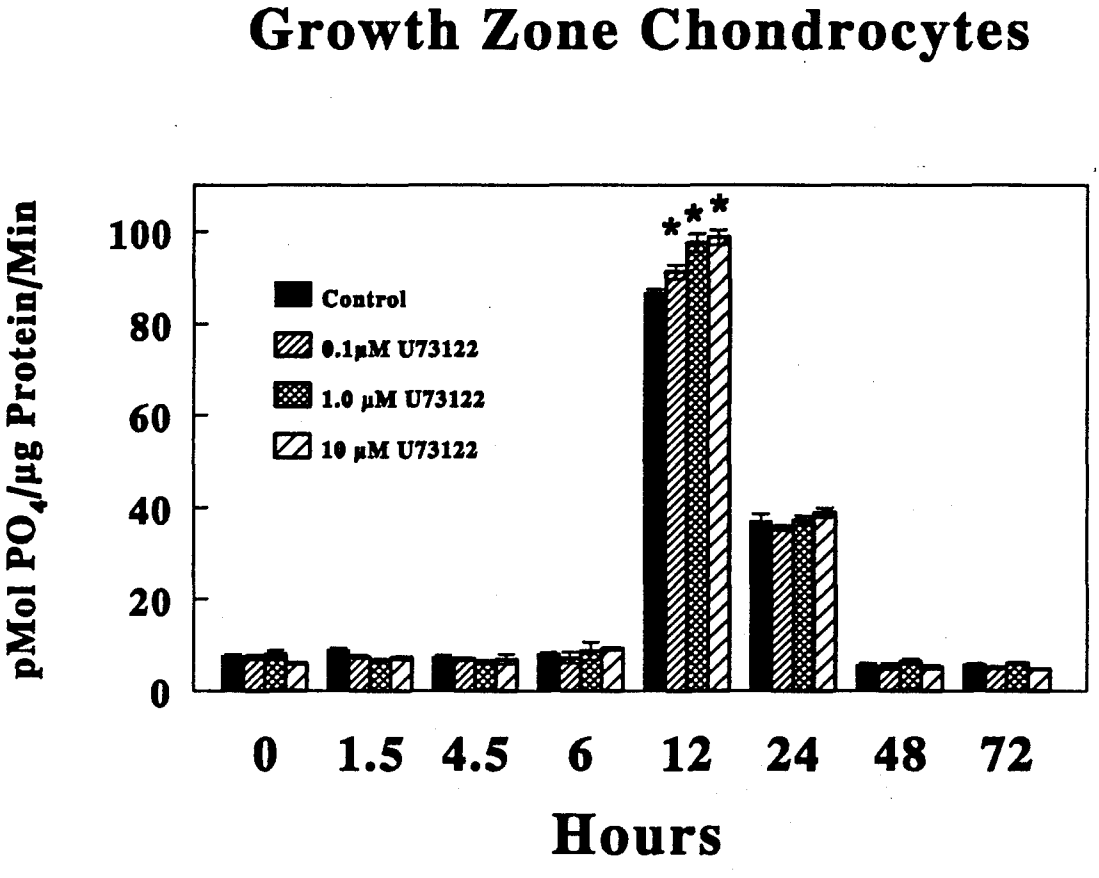
6. Tyrosine Kinase and Phospholipase C. The phospholipase C inhibitor U73122 (0.1-10μM) had no significant effect on basal PKC specific activity levels in confluent growth zone or resting zone chondrocytes treated with control DMEM + 10% FBS for 1.5, 4.5, 6, 12, 24, 48 or 72 hours (data not shown). In growth zone chondrocytes, U73122 had no dramatic affect on the TGF-β-induced rise in PKC specific activity observed at 12 or 24 hours following growth factor and phospholipase C inhibitor addition (Figure 9A). However, slight, but significant, increases in activity were found 12 hours after addition of rhTGF-β1 to growth zone chondrocytes cultured in the presence of 0.1-10μM U73122. This increase

Table 6. TRANSLOCATION OF PROTEIN KINASE C SPECIFIC ACTIVITY IN CHONDROCYTE CULTURES TREATED WITH rhTGF-β1.

	Growth Zone Cells		Resting Zone Cells	
Treatment	Membrane	Cytosol	Membrane	Cytosol
Control/PBS	0.240 ± 0.008	0.706 ± 0.001	0.229 ± 0.008	0.818 ± 0.011
0.11ng/ml TGFβ	0.562 ± 0.011#	1.369 ± 0.016*	0.690 ± 0.019#	2.784 ± 0.028*
0.22ng/ml TGFβ	0.406 ± 0.013	2.947 ± 0.020*	0.401 ± 0.010	4.955 ± 0.068*
10ng/ml TGFβ	0.312 ± 0.003	5.247 ± 0.019*	0.391 ± 0.011	13.291 ± 0.097*

Values represent the mean ± SEM protein kinase C specific activity (pMol PO_4/μg protein/min) of six cultures. Growth zone and resting zone chondrocytes were treated with rhTGF-β1 (TGFβ) for 12 hours. Control media contained phosphate buffered saline (PBS) vehicle. Data are from one of two replicate experiments. *P<0.01, treatment vs. control; #P<0.05, treatment vs. control; N.D., not done.

Figure 9A. EFFECT OF U73122 ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage chondrocytes were pretreated for 2 hours with 0-10 μM U73122, followed by fresh medium containing 5 ng/ml rhTGF-β1 and 0-10 μM U73122 for various times. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of two replicate experiments. *P<0.05, treatment vs. control.


Growth Zone Chondrocytes
pMol PO4/µg Protein/Min
100
80
60
40
20
0
Control
0.1µM U73122
1.0 µM U73122
10 µM U73122
* * *
0
1.5
4.5
6
12
24
48
72
Hours

appeared to be dose-dependent at 0.1 and 1μM U73122, but no further increase was seen at 10μM. For rhTGF-β1-treated resting zone cells, U73122 had no significant affect on PKC specific activity (Figure 9B). Unlike the slight enhancement seen in growth zone chondrocytes under similar conditions, no significant alteration of PKC specific activity was observed at the 6, 12, or 24 hour time points.

The tyrosine kinase inhibitor, genistein (0.1-10 μM), had no effect on basal PKC specific activity in growth zone or resting zone chondrocytes (data not shown). In growth zone chondrocytes, genistein had no effect on the rhTGF-β1-mediated increase in PKC specific activity at any of the time points examined (Figure 10A). In resting zone cells, genistein did not affect the TGFβ effect on PKC specific activity, but produced a small, but significant, increase at 12 hours for the 10μM dose (Figure 10B).

7. Transcription and Translation. Actinomycin D produced a dose-dependent inhibition of rhTGF-β1-stimulated PKC specific activity in growth zone chondrocytes at 12 hours (Figure 11A). In growth zone cultures treated with 0.11 ng/ml rhTGF-β1, the PKC specific activity was reduced 31.9% and 67.0% by 0.01 mM and 0.1 mM actinomycin D, respectively. In growth zone cultures treated with 0.22 ng/ml and 10 ng/ml rhTGF-β1, the PKC specific activity was similarly reduced. In resting zone cultures treated with 0.11 ng/ml rhTGF-β, the PKC specific activity was reduced by 41.6% and 71% by 0.01 mM and 0.1 mM actinomycin D, respectively (Figure 11B). In resting zone cultures treated with 0.22 ng/ml and 10 ng/ml rhTGF-β, the PKC specific activity was similarly reduced.

Cycloheximide also produced dose-dependent inhibition of rhTGF-β1-stimulated PKC specific activity in growth zone chondrocytes at 12 hours (Figure 12A). In growth zone cultures treated with 0.11 ng/ml rhTGF-β1, the PKC specific activity was reduced 42.7% and 73.3% by 0.01 mM and 0.1 mM cycloheximide, respectively. In growth zone cultures treated with 0.22 ng/ml and 10 ng/ml rhTGF-β1, the PKC specific activity was similarly, reduced. In

Figure 9B. EFFECT OF U73122 ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage chondrocytes were pretreated for 2 hours with 0-10 μM U73122, followed by fresh medium containing 5 ng/ml rhTGF-β1 and 0-10 μM U73122 for various times. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of two replicate experiments. *P<0.05, treatment vs. control.

Resting Zone Chondrocytes

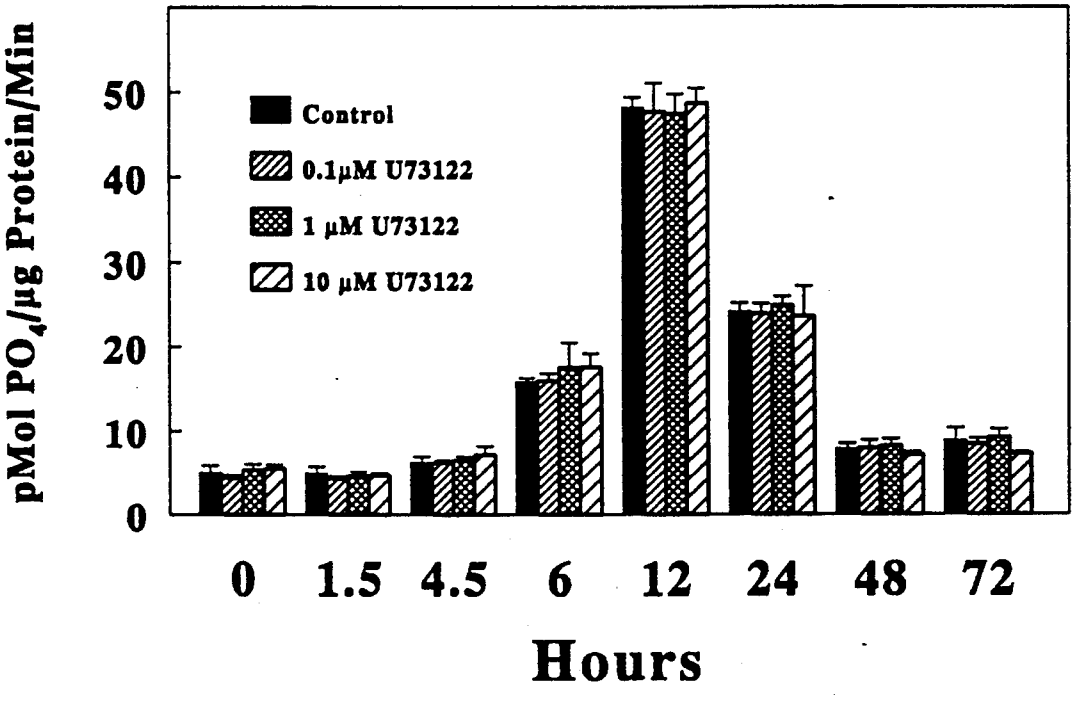


Figure 10A. EFFECT OF GENISTEIN ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage growth zone chondrocytes were pretreated for 2 hours with 0-10 μM genistein, followed by fresh medium containing 5 ng/ml rhTGF-β1 and 0-10 μM genistein for various times. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of two replicate experiments. *$P<0.05$, treatment vs. control.

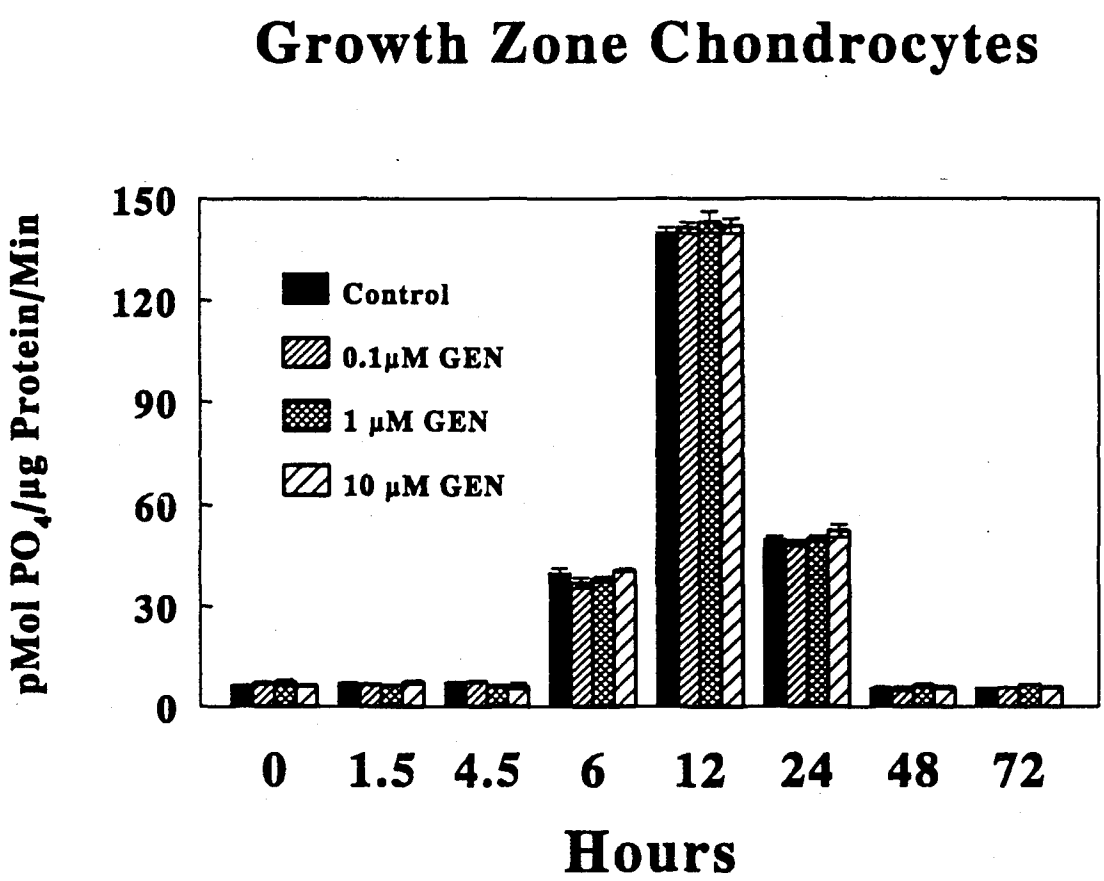

Growth Zone Chondrocytes
pMol PO4/µg Protein/Min
150
120
90
60
30
0
Control
0.1µM GEN
1 µM GEN
10 µM GEN
0
1.5
4.5
6
12
24
48
72
Hours

Figure 10B. EFFECT OF GENISTEIN ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage resting zone chondrocytes were pretreated for 2 hours with 0-10 μ M genistein, followed by fresh medium contaning 5 ng/ml rhTGF-β1 and 0-10 μM genistein for various times. Each point represents the mean ± SEM of protein kinase C specific activity for six cultures. Data are from one of two replicate experiments. *P<0.05, treatment vs. control.

Resting Zone Chondrocytes

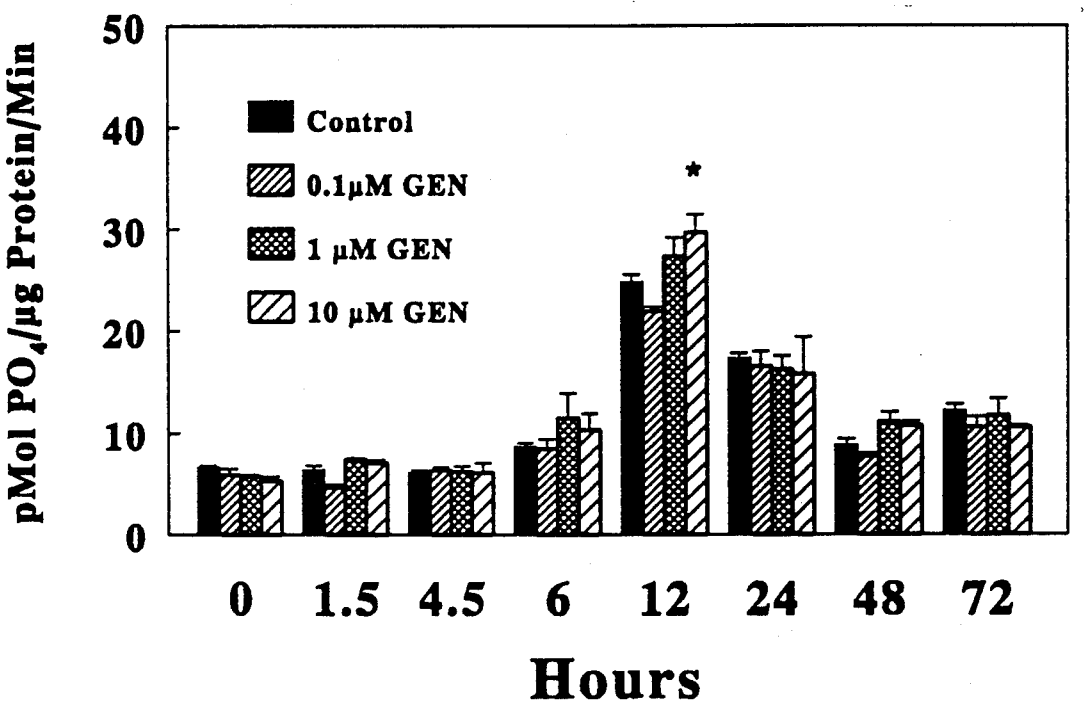


Figure 11A. EFFECT OF ACTINOMYCIN D ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage cultures were treated for 12 hours with various concentrations of actinomycin D with or without 0.11 ng/ml, 0.22 ng/ml, or 10 ng/ml rhTGF-β1. Data shown are the mean ± SEM for 6 cultures. *$p<0.05$. To ensure validity, experiments were repeated a minimum of 3 times.

Growth Zone Chondrocytes

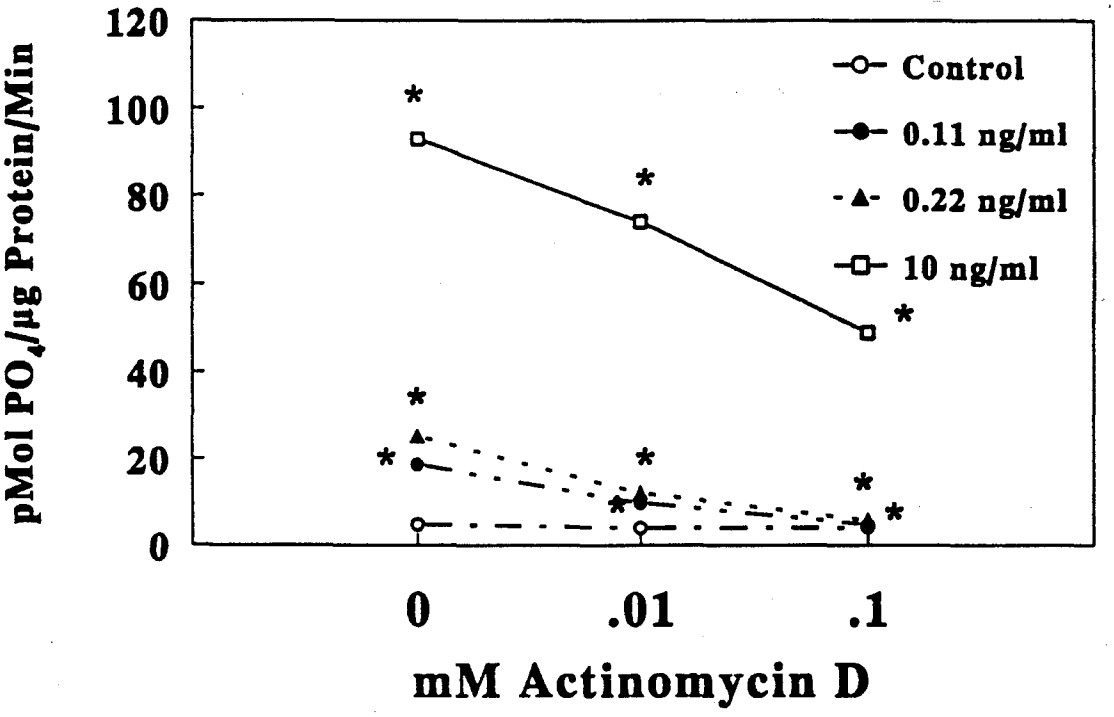


Figure 11B. EFFECT OF ACTINOMYCIN D ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage cultures were treated for 12 hours with various concentrations of actinomycin D with or without 0.11 ng/ml, 0.22 ng/ml, or 10 ng/ml rhTGF-β1. Data shown are the mean ± SEM for 6 cultures. *$p<0.05$. To ensure validity, experiments were repeated a minimum of 3 times.

Resting Zone Chondrocytes

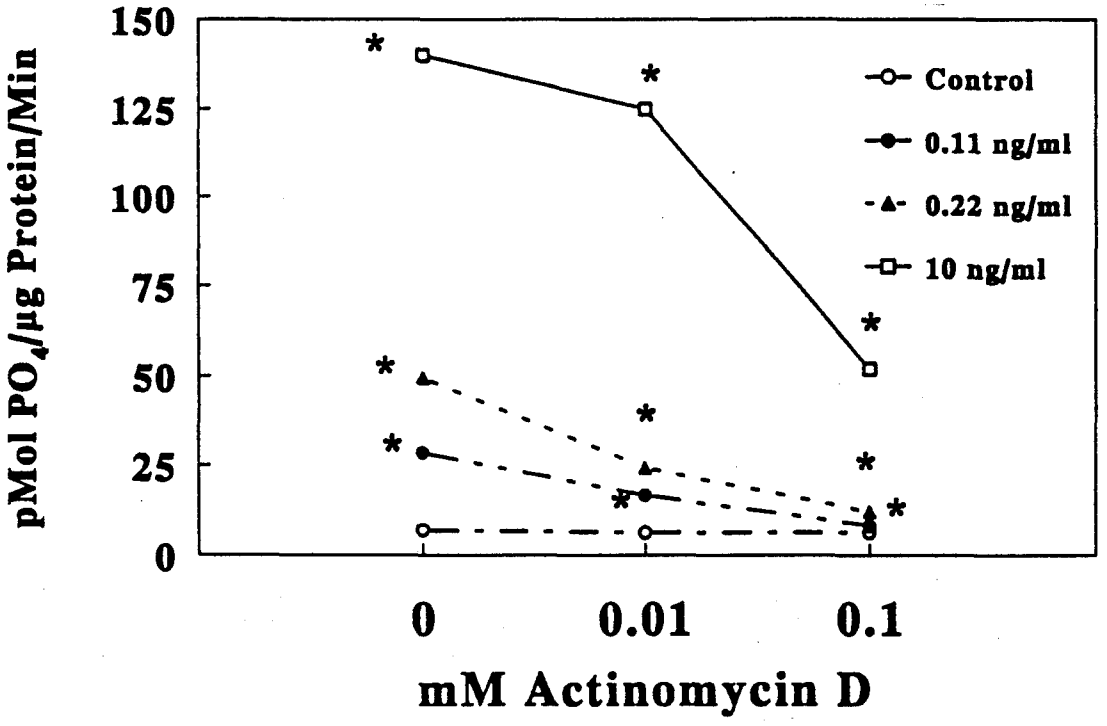
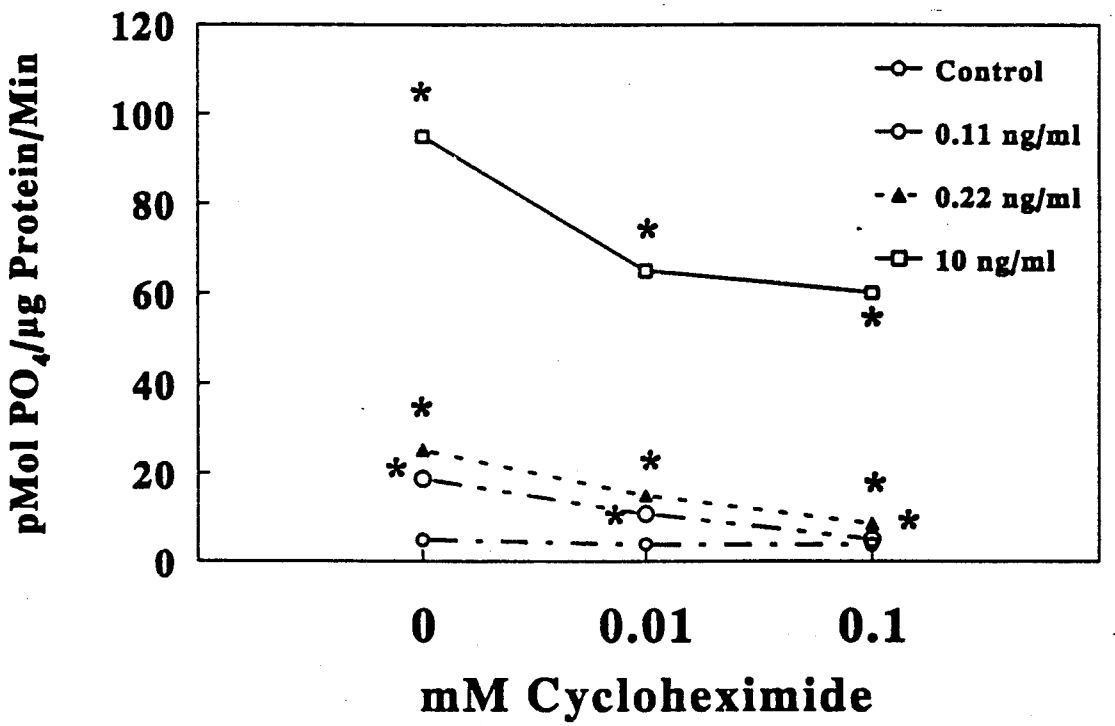


Figure 12A. EFFECT OF CYCLOHEXIMIDE ON PROTEIN KINASE C SPECIFIC ACTIVITY IN GROWTH ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage cultures were treated for 12 hours with various concentrations of cycloheximide with or without 0.11 ng/ml, 0.22 ng/ml, or 10 ng/ml rhTGFβ_1. Data shown are the mean $\pm$ SEM for 6 cultures. *$p<0.05$. To ensure validity, experiments were repeated a minimum of 3 times.

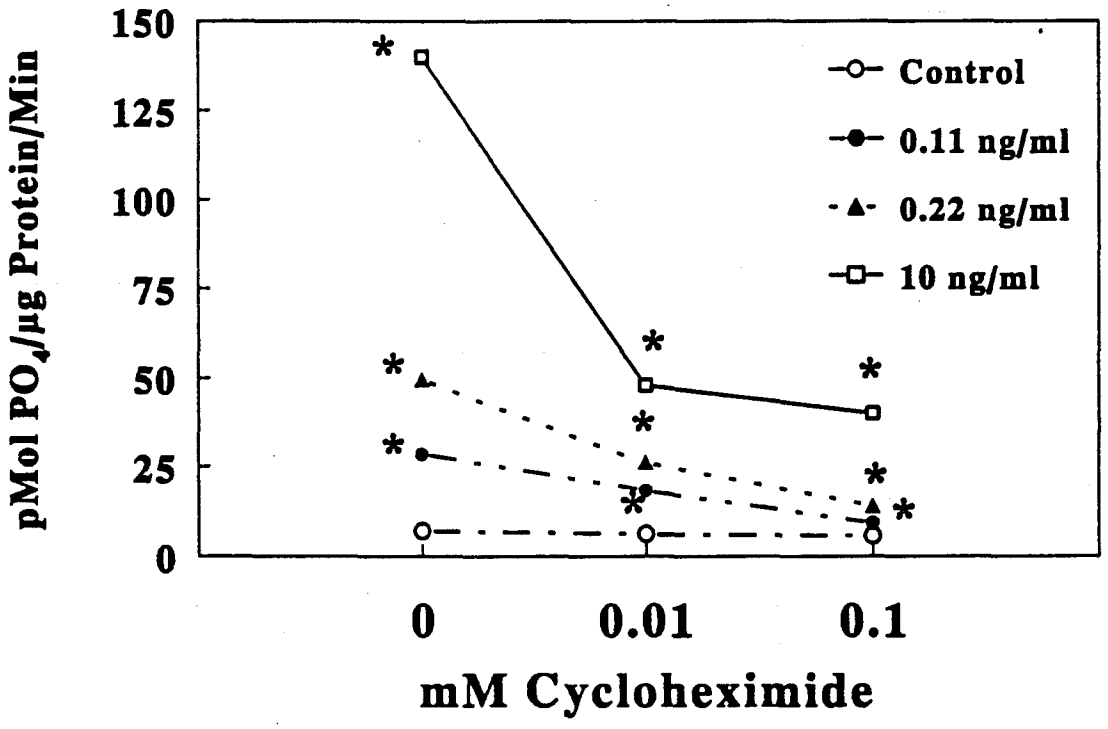
Growth Zone Chondrocytes



resting zone cultures treated with 0.11 ng/ml rhTGF-β1, the PKC specific activity was reduced 35.9% and 67.7% by 0.01 mM cycloheximide and 0.1 mM cycloheximide, respectively (Figure 12B). In resting zone cultures treated with 0.22 ng/ml and 10 ng/ml rhTGF-β1, the PKC specific activity was similarly reduced.

Figure 12B. EFFECT OF CYCLOHEXIMIDE ON PROTEIN KINASE C SPECIFIC ACTIVITY IN RESTING ZONE CHONDROCYTES TREATED WITH rhTGF-β1. Confluent fourth passage cultures were treated for 12 hours with various concentrations of cycloheximide with or without 0.11 ng/ml, 0.22 ng/ml, or 10 ng/ml rhTGFβ_1. Data shown are the mean $\pm$ SEM for 6 cultures. *$p<0.05$. To ensure validity, experiments were repeated a minimum of 3 times.

Resting Zone Chondrocytes



IV. DISCUSSION

Protein kinase C specific activity is elevated in rat costochondral chondrocytes incubated with vitamin D_3 metabolites; however, the characteristics are distinct with respect to the factor used or the type of cell. Response to vitamin D_3 metabolites is dependent upon cell maturation, with growth zone and resting zone cultures displaying differential sensitivity and response time to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, respectively.

Specifically, these studies have revealed a rapid increase in PKC specific activity in 1,25-$(OH)_2D_3$-treated growth zone chondrocytes and a relatively slower increase in 24,25-$(OH)_2D_3$-treated resting zone chondrocytes. The translocation experiment demonstrated that exposure to 1,25-$(OH)_2D_3$ elicited an increase in membrane-bound PKC specific activity in growth zone cells, and exposure to 24,25-$(OH)_2D_3$ produced an increase in membrane-bound PKC specific activity in resting zone cells. In many cell types, activation of protein kinase C by hormones or growth factors is associated with rapid translocation of protein kinase C from the cytosol to membrane components (Abou-Samra, *et al.*, 1989, Hirota *et al.*, 1985, Ohno *et al.*, 1991). 24,25-$(OH)_2D_3$ had no effect on PKC specific activity in growth zone cells, and 1,25-$(OH)_2D_3$ had no effect on PKC specific activity in resting zone cells. The cell specificity of the results supports the concept that 1,25-$(OH)_2D_3$ targets growth zone cells, whereas 24,25-$(OH)_2D_3$ targets resting zone cells.

The type of protein kinase that was stimulated by the vitamin D metabolites was the Ca^{+2}/phospholipid-dependent enzyme, as demonstrated by the inhibitor studies discussed below. This indicates that changes in phospholipid metabolism and Ca flux are involved. Previous studies have shown that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ regulate arachidonic acid incorporation and release, as well as ^{45}Ca flux (Langston, *et al.*, 1990, Schwartz, *et al.*, 1990, Swain *et al.*, 1992). Furthermore, effects of these metabolites on phospholipase A_2 activity (Schwartz *et al.*, 1988) and membrane fluidity (Swain *et al.*, 1993) are directly on the

membrane and therefore nongenomic. Thus, it is likely that the vitamin D_3 metabolite effects on protein kinase C include nongenomic mechanisms as well.

To investigate the precise mechanisms of signal transduction utilized by the vitamin D_3 metabolites, the potential involvement of phospholipase C and tyrosine kinases were assessed. The aminosteroid compound U73122 has been shown to specifically inhibit phospholipase C and to have no effect on phospholipase A_2, by interacting directly with the phospholipase C enzyme itself (Bleasdale *et al.*, 1989), making it a useful reagent for determination of phospholipase C-mediated events. U73122 reduced 1,25-$(OH)_2D_3$-stimulated PKC specific activity in growth zone cell cultures in a dose-dependent manner, yet had no effect on the 24,25-$(OH)_2D_3$ response. This result suggests that signaling by 1,25-$(OH)_2D_3$ is phospholipase C dependent, while 24,25-$(OH)_2D_3$ may activate protein kinase C by an alternative pathway.

The failure of genistein to inhibit the chondrocyte protein kinase C response to 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ simply suggests that tyrosine kinases are not involved in the protein kinase C-dependent pathways of these factors.

The contention that the vitamin D_3 metabolites activate protein kinase C via distinct modes of action is strongly supported by the fact that actinomycin D and cycloheximide have no effect on 1,25-$(OH)_2D_3$-stimulated activity in growth zone chondrocytes, while both inhibit 24,25-$(OH)_2D_3$-induced activity in resting zone chondrocytes. The data suggest that 1,25-$(OH)_2D_3$ signals through a nongenomic pathway, whereas 24,25-$(OH)_2D_3$ signal through a genomic pathway with respect to protein kinase C-mediated events. Other mechanisms of 24,25-$(OH)_2D_3$ action on resting zone chondrocytes do involve nongenomic events (Swain *et al.*, 1992, 1993, Schwartz *et al.*, 1988).

The rapid effect of 1,25-$(OH)_2D_3$ on protein kinase C in growth zone chondrocytes suggests that it directly transduces its actions at least in part via a nongenomic mechanism. Of course, nongenomic activation of protein kinase C may subsequently produce genomic effects.

Others have shown that inhibition of PKC specific activity blocks 1,25-$(OH)_2D_3$-induced osteocalcin production in osteoblasts (van Leeuwen *et al.*, 1992), indicating a protein kinase C-dependent mechanism for 1,25-$(OH)_2D_3$ signaling in bone as well. 1,25-$(OH)_2D_3$ increases inositol triphosphate and diacylglycerol levels in osteoblasts (Civitelli *et al.*, 1990), suggesting a phospholipase C-dependent mechanism. Morever, 1,25-$(OH)_2D_3$ can activate Ca^{+2} channels in the intestine in a process referred to as "transcaltachia" (de Boland and Norman, 1990), and protein kinase C is known to activate Ca^{+2} channels.

It is likely that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have distinct functions in cartilage and transduce their transmembrane signals via distinct pathways. A primary function of 1,25-$(OH)_2D_3$ is to promote Ca transport (Lieberherr *et al.*, 1989), whereas 24,25-$(OH)_2D_3$ appears to be involved in promoting early differentiation events (de Boland and Nemere, 1992). Thus, a phospholipase C-dependent mechanism, with consequent Ca^{+2} transport, is not a feature of the 24,25-$(OH)_2D_3$-dependent protein kinase activation. The 90 minute delay in enzyme activation by 24,25-$(OH)_2D_3$ suggests that its effects are subsequent to other regulatory events in the cell. 24,25-$(OH)_2D_3$ stimulates 1,25-$(OH)_2D_3$ production by resting zone cells (Schwartz *et al.*, 1992), causing a significant increase in 1,25-$(OH)_2D_3$ at one hour. While vitamin D metabolism may not account for the stimulation of PKC specific activity observed, it does demonstrate the complex interrelationship of these factors and others in chondrocyte regulation.

These experiments are consistent with the emerging realization that the classical steroid hormone receptor-mediated pathway is not sufficient to explain all of the known effects of vitamin D_3. Specifically, the action of vitamin D_3 metabolites on PKC specific activity in cultured chondrocytes is rapid and dependent upon the differentiation state of the chondrocytes.

rhTGF-β1 increases PKC specific activity in rat costochondral chondrocytes, both in the less differentiated resting zone cells and in the more differentiated growth zone cells. Fold-

increases in PKC specific activity generally varied from 5-30% between experiments; this fluctuation may represent differences in TGF-β receptor number or other aspects of cell maturation within each batch of primary cell cultures. Inhibitor studies verify that the kinase activity activated by TGF-β is in fact protein kinase C. The immunoprecipitation experiments revealed that protein kinase C alpha is the major isoform present in untreated and rhTGF-β1-treated resting zone cells. PKC specific activity in lysates from control and rhTGF-β1-treated cells was unaffected by antibodies specific for the beta, delta and epsilon isoenzymes, but significantly reduced by the antibody against the alpha isoenzyme. PKC specific activity in the TGF-β-treated cell lysates was decreased to a greater extent than in the control cells.

Unlike the more rapid responses observed for vitamin D_3 metabolites, rhTGF-β1 elicits a dose-dependent increase in chondrocyte PKC specific activity only after six hours of exposure in resting zone cells and nine hours of exposure in growth zone cells. In both cell types, peak PKC specific activity was observed at 12 hours following rhTGF-β1 exposure; however, the increase in activity began sooner and was sustained longer in resting zone chondrocytes. This is in contrast to the peaks at nine minutes for 1,25-$(OH)_2D_3$ in growth zone chondroyctes and 90 minutes for 24,25-$(OH)_2D_3$ in resting zone chondrocytes.

The time course of protein kinase C activation by rhTGF-β1 in rat costochondral chondrocytes is not consistent with classical signal transduction events, which occur within minutes rather than hours. By comparison, 250pM TGF-β1 has been shown to elicit more rapid protein kinase C-dependent responses in mink lung epithelial cells (Ohtsuki and Massague, 1992). In these cells, TGF-β1 induced expression of *junB* and plasminogen activator inhibitor-1 within 90 minutes, and their expression was completely blocked by the protein kinase C inhibitor H7. In vascular smooth muscle cells, 4×10^{-11} M TGF-β1 elicited membrane translocation and activation of protein kinase C within 15 minutes (Wrenn *et al.*, 1992). Our results demonstrate no measurable effect of rhTGF-β1 upon chondrocyte PKC specific activity before at least six hours of exposure. This observation is consistent with the

effect of 10 ng/ml TGF-β1 on phosphoinositide metabolism in Rat-1 cells (Muldoon *et al.*, 1988), in which inositol trisphosphate levels peaked at six hours and remained elevated at eight hours. Interestingly, and also consistent with our findings, the effect of TGF-β1 upon inositol phosphate levels was inhibited by actinomycin D.

The long time course of rhTGF-β1 action on protein kinase C in growth zone and resting zone chondrocytes is different from other polypeptide growth factors, which often utilize rapid phospholipase C or tyrosine kinase-dependent pathways. The insensitivity to the phospholipase C inhibitor U73122 suggests that the rhTGF-β1-induced PKC specific activity does not require the action of phospholipase C on membrane phospholipids. The rhTGF-β1 was co-administered with U73122 following a two-hour pretreatment with U73122 alone, and the inhibitor remains active for at least six to eight hours (Bleasdale *et al.*, 1989). If a phospholipase C-dependent event were required, there would have been a reduction in PKC specific activity, yet no significant inhibition was observed. On the contrary, in growth zone cells, significant increases in PKC specific activity was observed at 12 hours for cultures treated with rhTGF-β1 and U73122 simultaneously.

Other pathways which utilize protein kinase C are those initiated by tyrosine kinase transmembrane receptors such as those for epidermal growth factor (Mioh and Chen, 1989), insulin-like growth factors (Linkhart and Keffer, 1991), and platelet-derived growth factor (Battegay *et al.*, 1990). Upon ligand binding, the cytoplasmic tyrosine kinase domain of these receptors is activated, resulting in the tyrosine phosphorylation of effector molecules and signal propagation. Failure to detect any reduction in PKC specific activity, when either growth zone or resting zone chondrocytes was pretreated with genistein and administered rhTGF-β1 plus genistein, suggests that tyrosine kinases are not required for protein kinase C activation by rhTGF-β1 in costochondral chondrocytes. Autocrine production of platelet-derived growth factor or other tyrosine kinase-associated growth factors does not appear to be necessary for protein kinase C activation, as their actions would be inhibited by genistein.

Nevertheless, this does not rule out the possible involvement of TGFβ-induced growth factor secretion in mesenchymal cell proliferation, nor does it rule out the possiblity that TGF-β initiates a cascade of growth factor production and differentiation events (Battegay *et al.*, 1990, Majack *et al.*, 1990, Sporn *et al.*, 1987[1]).

Also consistent with the lack of early membrane-associated enzymatic effects for rhTGF-β1 is the observation of minimal translocation of protein kinase C to the cell membrane at the time of maximal stimulation. In fact, the higher dose of rhTGF-β1 produced less activity in the membrane fraction than the lower dose. This is in contrast to the 22.2-to-28.0-fold increase in translocation observed in growth zone chondrocytes treated with 1,25-$(OH)_2D_3$ or the 16.2-to-30.8-fold increases observed in resting zone cells treated with 10^{-9}-10^{-8} M 24,25-$(OH)_2D_3$. While substantial translocation was not evident, cytosolic PKC specific activity was significantly elevated at 12 hours, suggesting a distinct mechanism of activation by rhTGF-β1 compared to that seen with the vitamin D metabolites. The dose-dependent increase in cytosolic, rather than membrane, protein kinase C, suggests that TGFβ may stimulate basal enzyme production via a genomic mechanism. Subsequently, other agents, acting through nongenomic mechanisms, may enhance membrane translocation of this increased reserve of protein kinase C. It is also possible that at later time points the elevated cytosolic enzyme would have been translocated to the membrane.

Our results indicated major differences in the time course, biochemical pathway, and subcellular localization of protein kinase C activation by rhTGF-β1, all of which are consistent with a genomic, rather than a nongenomic, effect. The Western blot also suggests that rhTGF-β1 treatment may increase the relative amount of protein kinase C protein present in the culture, indicating a genomic mechanism of action. Interestingly, the modest increase in band intensity observed is apparently sufficient for the substantial increase in activity obtained. This hypothesis was supported by the reduction of PKC specific activity observed in cultures treated with rhTGF-β1 in conjunction with the transcription inhibitor actinomycin D or the

translation inhibitor cycloheximide. When compared at equimolar amounts, actinomycin D always produced slightly more inhibition than cycloheximide in both types of chondrocytes. These data strongly suggest that *de novo* RNA and protein synthesis are both required for TGFβ-induced PKC specific activity.

In summary, these experiments have shown that PKC specific activity is regulated by TGFβ in costochondral chondrocytes in a time-dependent and cell maturation specific manner. The mechanism of activation does not appear to involve either phospholipase C or tyrosine kinase activity; however, new gene transcription and translation are involved. The results suggest that the TGFβ effects on protein kinase C are the result of a regulatory cascade. Increased cytosolic enzyme activity may serve as a reservoir for non-genomic translocation of protein kinase C in response to other regulatory factors or as a downstream event in the TGF β-dependent stimulation of the enzyme.

LITERATURE CITED

Abou-Samra, A., Jueppner, H., Westerberg, D., Potts, J. and Segre, G. (1989) Parathyroid hormone causes translocation of protein kinase C from cytosol to membranes in rat osteosarcoma cells. Endocrinology, **124**:1107-1113.

Anderson, H.C. (1969) Vesicles associated with calcification in the matrix of epiphyseal cartilage. J. Cell. Biol., **41**:59-72.

Anderson, H. and Sajdera, S. (1976) Calcification of rachitic cartilage to study matrix vesicle function. Fed. Proc., **35**:148-153.

Atkin, I., Pita, J., Ornoy, A., Agundez, A., Castiglione, G. and Howell, D. (1985) Effects of vitamin D metabolites on healing of low phosphate, vitamin D-deficient induced rickets in rats. Bone, **6**:113-123.

Balsan, S., Garabedian, M., Larchet, M., Gorski, A., Cournot, G., Tau, C., Bourdeau, A., Silve, C. and Ricour, C. (1986) Long-term nocturnal calcium infusions can cure rickets and promote normal mineralization in hereditary resistence to 1,25-dihydroxyvitamin D. J. Clin. Invest., **77**:1661-1667.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F. and Ross, R. (1990) TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell, **63**:515-524.

Beck, L.S., Deguzman, L., Lee, W.P., Xu, Y., McFatridge, LA., Gillet, N.A. and Amento, E.P. (1991) TGF-beta 1 induces bone closure of skull defects. J. Bone Min. Res., **6**:1257-1265.

Bell, R.M., Hannun, Y. and Loomis, C. (1986) Mixed micelle assay of protein kinase C. Methods Enzymol., **124**:353-356.

Beresford, J., Gallagher, J. and Russell, R. (1986) 1,25-Dihydroxyvitamin D_3 and human bone-derived cells *In Vitro* and effects on alkaline phosphatase, type I collagen and proliferation. Endocrinology, **119**:1776-1785.

Bernstein, E.F., Harisiadis, L., Salomon, G., Norton, J., Sollberg, S., Uitto, J., Glastein, E., Glass, J., Talbot, T., Russo, A. and Mitchell, J.B. (1991) Transforming growth factor-beta improves healing of radiation-impaired wounds. J. Invest. Dermatol., **97**:430-434.

Berridge, M.J. (1987) Inositol lipids and cell proliferation. Biochim. Biophys. Acta., **907**:33-45.

Blumberg, P.M. (1980) In Vitro studies on the mode of action of the phorbol esters, potent tumor promoters: Part I. CRC Crit. Rev. Tox., **8**:153-197.

Bonewald, L.F., Kester, M., Schwartz, Z., Swain, L.D., Khare, A., Johnson, T.L., Leach, R.J. and Boyan, B.D. (1992) Effects of combining transforming growth factor beta and 1,25-dihydroxyvitamin D_3 on differentiation of a human osteosarcoma (MG-63). J. Biol. Chem., **267**:8943-8949.

Bonewald, L.F., Schwartz, Z., Swain, L.D., Ramirez, V., Poser, J. and Boyan, B.D. (1990) Stimulation of plasma membrane and matrix vesicle activity by transforming growth factor-beta in osteosarcoma cell cultures. J. Cellular Phys., **145**:200-206.

Boskey, A.L. (1981) Current concepts of the physiology and biochemistry of calcification. Clin. Orthop. Rel. Res., **157**:225-255.

Bourdeau, A., Atmani, F., Grosse, B. and Lieberrherr, M. (1990) Rapid effects of 1,25-dihydroxyvitamin D_3 and extracellular Ca^{2+} on phospholipid metabolism in dispersed porcine parathyroid cells. Endocrinology, **127**:2738-2743.

Boyan, B.D., Schwartz, Z., Swain, L.D., Carnes, D.L. and Zislis, T. (1988) Differential expression of phenotype by resting zone and growth region costochondral chondrocytes In Vitro. Bone, **9**:185-194.

Boyan, B.D., Schwartz, Z. and Swain, L.D. (1992) In vitro studies on the regulation of endochondral ossification by vitamin D. Critical Reviews in Oral Biol. and Med., **3(1/2)**:15-30.

Brighton, C. and Hunt, R. (1976) Histochemical localization of calcium in growth plate mitochondria and matrix vesicles. Fed Proc., **35**:143-147.

Canalis, E. and Lian, J. (1985) 1,25-Dihydroxyvitamin D_3 effects on collagen and DNA synthesis in periosteum and periosteum-free calvaria. Bone, **6**:457-460.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem., **257**:7847-7851.

Cecil, R. and Anderson, H. (1978) Freeze-fracture studies of matrix vesicle calcification in epiphyseal growth plate. Metab. Bone Dis., **1**:89-93.

Chakrabarty, S. (1992) Role of protein kinase C in transforming growth factor-beta1 induction of carcinoembryonic antigen in human colon carcinoma cells. J. Cell. Phys., **152**:494-499.

Chen, T.L., Bates, R.L., Xu, Y., Ammann, A.J. and Beck S. (1992) Human recombinant transforming growth factor-beta1 modulation of biochemical and cellular events in healing of ulcer wounds. J. Invest. Dermatol., **98**:428-435.

Civitelli, R., Kim, Y.S., Gunsten, S.L., Fujimori, A., Huskey, M., Avioli, L.V. and Hruska, K.A. (1990) Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology, **127**:2253-2262.

de Boland, A.R. and Nemere, I. (1992) Rapid actions of vitamin D compounds. J. Cell. Biochem., **49**:32-36.

de Boland, A.R. and Norman, A.W. (1990) Evidence for involvement of protein kinase C and cyclic adenosine 3', 5' monophosphate-dependent protein kinase in the 1,25-dihydroxyvitamin D_3-mediated rapid stimulation of intestinal calcium transport, (transcaltachia). Endocrinology, **127**:39-45.

DeLarco, J. and Todaro, G. (1978) Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci., USA, **75**:4001-4005.

Felig, P., Baxter, J.D., Broadus, A.E. and Frohman, L.A. (1981) In: Endocrinology and Metabolism. New York: McGraw-Hill, pp. 63-105.

Glaser, J. and Conrad, H. (1981) Formation of matrix vesicles by cultured chick embryo chondrocytes. J. Biol. Chem., **256**:12607-12611.

Glowacki, J. (1982) In: Factors and mechanisms influencing bone growth. New York: Alan R. Liss, Inc. pp. 83-91.

Graves, D.T. and Cochran, D.L. (1990) Mesenchymal cell growth factors. Oral Biol. and Med., **1**:17-27.

Hale, L., Kemick, M. and Wuthier, R. (1986) Effect of vitamin D metabolites on the expression of alkaline phosphatase activity by epiphyseal hypertrophic chondrocytes in primary cell culture. J. Bone Min. Res., **1**:489-495.

Hirota, K., Hirota, T., Aguilera, G. and Catt, K. (1985) Hormone-induced redistribution of calcium-activated phospholipid-dependent protein kinase in pituitary gonadotrophs. J. Biol. Chem., **260**:3243-3247.

Joyce, M., Roberts, A., Sporn, M. and Bolander, M. (1990) Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell. Biol., **110**:2195-2207.

Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y. (1980) Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. Biochem. Biophys. Res. Commun., **97**:309-317.

Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K. and Nishizuka, Y. (1987) Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. FEBS Lett., **217**:227-231.

Knopf, J.L., Lee, M.H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M. and Bell, R.M. (1986) Cloning and expression of multiple protein kinase C cDNAs. Cell, **46**:491-502.

Ksiazek, T. and Moskalewski, S. (1983) Studies on bone formation by cartilage reconstructed by isolated epiphyseal chondrocytes, transplanted syngeneically or across known histocompatability barriers in mice. Clin. Orthop. Rel. Res., **172**:233-242.

Langston, G.G., Swain, L.D., Schwartz, Z., Del Toro, F., Gomez, R. and Boyan, B.D. (1990) Effect of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ on calcium fluxes in costochondral chondrocyte cultures. Calcif. Tissue Int., **47**:230-236.

Lehninger, A.L. (1982) In: Principles of Biochemistry. New York: Worth Publishers, pp.377-380.

Lieberherr, M., Grosse, B., Duchambon, P. and Drueke, T. (1989) A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D_3 on the phosphoinositide metabolism in rat enterocytes. J. Biol. Chem., **264**:20403-20406.

Linkhart, T.A. and Keffer, M.J. (1991) Differential regulation of insulin-like growth factor-I (IGF-I) and IGF-II release from cultured neonatal mouse calvaria by parathyroid hormone, transforming growth factor-beta, and 1,25-dihydroxyvitamin D_3. Endocrinology, **128**:1511-1518.

Majack, R.A., Majesky, M.W. and Goodman, L.V. (1990) Role of PDGF-A expression in the control of vascular smooth muscle growth by TGFβ. J. Cell. Biol., **111**:239-247.

Majeska, R. and Rodan, G. (1982) The effect of 1,25-$(OH)_2D_3$ on alkaline phosphatase in osteoblastic osteosarcoma cells. J. Biol. Chem., **257**:3362-3365.

McLaughlin, J.A., Cantley, L.C., Chahwala, S.B. and Holick, M.F. (1987) 1,25-$(OH)_2D_3$ increases intracellular calcium in human keratinocytes by stimulating phosphatidylinositol turnover. J. Bone Min. Res., (abst.), **2**:76.

Mioh, H. and Chen, J.K. (1989) Differential inhibitory effects of TGF-beta on EGF-, PDGF- and HBGF-1-stimulated MG63 human osteosarcoma cell growth: possible involvement of

growth factor interactions at the receptor and postreceptor levels. J. Cell. Physiol., **139**:509-516.

Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988) Transforming growth factor beta and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in rat-1 fibroblasts. J. Biol. Chem., **263**:5030-5033.

Mustoe, T.A., Pierce, G.F., Thomason, A., Gramates, P., Sporn, M.B. and Deuel, T.F. (1987) Accelerated healing of incisional wounds in rats induced by transforming growth factor-β. Science, **237**:1333-1336.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature, **308**:693-698.

Obeid, L.M., Okazaki, T., Karolak, L.A. and Hannun, Y.A. (1990) Transcriptional regulation of protein kinase C by 1,25-dihydroxyvitamin D_3 in HL-60 cells. J. Biol. Chem., **265**:2370-2374.

Ohno, S., Akita, Y., Hata, A., Osada, S., Kubo, K., Konno, Y., Akimoto, K., Mizuno, K., Saido, T. and Kuroki, T. (1991) Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family; two distinct classes of PKC, conventional cPKC and novel nPKC. Adv. Enzyme Reg., **31**:287-303.

Ohtsuki, M. and Massague, J. (1992) Evidence for the involvement of protein kinase activity in transforming growth factor-beta signal transduction. Molecular and Cellular Biology, **12**:261-265.

Oshima, J., Watanabe, M., Hirosumi, J. and Orimo, H. (1987) 1,25-$(OH)_2D_3$ increases cytosolic Ca^{2+} concentration of osteoblastic cells, clone MC3T3-E1. Biochem. Biophys. Res. Commun., **145**:956-960.

Parker, P.J., Gurdip K., Marais, R.M., Mitchell, F., Pears, C., Schaap. D., Stabel, S. and Webster, C. (1989) Protein kinase C-a family affair. Molecular and Cellular Endocrinology, **65**:1-11.

Pfeilschifter, J. and Mundy, G.R. (1987) Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones. Proc. Natl. Acad. Sci. U.S.A., **84**:2024-2028.

Raisz, L. and Kream, B. (1983) Regulation of bone formation. N. Eng. J. Med., **309**:83-89.

Rifas, L., Shen, V. and Mitchell, V. (1982) Selective emergence of differentiated chondrocytes during serum-free culture of cells derived from fetal rat calvaria. J. Cell. Biol., **92**:493-504.

Roberts, A., Lamb, L., Newton, D., Sporn, M., DeLarco, J. and Todaro, G. (1980) Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acid ethanol extraction. Proc. Natl. Acad. Sci. USA, **77**:3494-3498.

Roberts, A.B., Anzano, M.A., Meyers, C.A., Wideman, J., Blacher, R., Pan, Y., Stein, S., Lehrman, R., Smith, J.M., Lamb, L.C. and Sporn, M.B. (1983) Purification and properties of a type beta transforming growth factor from bovine kidney. Biochemistry, **22**:5692-5698.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Wakefield, L.M., Heine, U.I., Liotta, L.A., Kehrl, J.H. and Fauchi, A.S. (1986) Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci., **83**:4167-4171.

Rosier, R.N., O'Keefe, R.J., Crabb, I.D. and Puzas, J.E. (1989) Transforming growth factor beta: An autocrine regulator of chondrocytes. Connective Tissue Research, **20**:295-301.

Schwartz, Z., Brooks, B., Swain, L.D., Del Toro, F., Norman, A. and Boyan, B.D. (1992) Production of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 by growth zone and resting zone chondrocytes is dependent on cell maturation and is regulated by hormones and growth factors. Endocrinology, **130**:2495-2504.

Schwartz, Z. and Boyan, B.D. (1988) The effects of vitamin D metabolites on phospholipase A_2 activity in growth zone and resting zone cartilage cells *In Vitro*. Endocrinology, **122**: 2191-2198.

Schwartz, Z., Knight, G., Swain, L. and Boyan, B.D. (1988) Localization of vitamin D_3-responsive alkaline phosphatase in cultured chondrocytes. J. Biol. Chem., **263**:6023-6026.

Schwartz, Z., Schlader, D.L., Ramirez, V., Kennedy, M.B. and Boyan, B.D. (1989) Effects of vitamin D metabolites on collagen production and cell proliferation of growth zone and resting zone cartilage cells *In Vitro*. J. Bone Min. Res., **4**:199-207.

Schwartz, Z., Schlader, D.L., Swain, L.D. and Boyan, B.D. (1988) Direct effects of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 on growth zone and resting zone chondrocyte membrane alkaline phosphatase and phospholipase-A_2 specific activities. Endocrinology, **123**:2878-2884.

Schwartz, Z., Swain, L., Kelly, D., Brooks, B. and Boyan, B. (1992) Regulation of prostaglandin E_2 production by vitamin D metabolites in growth zone and resting zone chondrocyte cultures is dependent on cell maturation. Bone, **13**:395-401.

Schwartz, Z., Swain, L.D., Ramirez, V. and Boyan, B.D. (1990) Regulation of arachidonic acid turnover by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in growth zone and resting zone chondrocyte cultures. Biochim. Biophys. Acta., **1027**:278-286.

Sporn, M., Roberts, A., Wakefield, L. and Assoian, R. (1986) Transforming growth factor beta biological function and chemical structure. Science, **233**:532-534.

Sporn, M.B., Roberts, A.B., Wakefield, L.M. and de Crombrugghe, B. (1987) Some recent advances in the chemistry and biology of transforming growth factor-beta. J. Cell. Biol., **105**:1039-1045.

Sutherland, E.W. and Robison, G.A. (1966) Metabolic effects of catecholamines. Pharmacol. Rev., **18**:145-161.

Suzuki, F., Takase, T., Takigawa, M., Uchida, A. and Shimomura, Y. (1981) Simulation of the initial state of endochondral ossification: Sequential culture of growth cartilage cells and bone marrow cells. Proc. Natl. Acad. Sci. USA, **78**:2368-2372.

Swain, L.D., Schwartz, Z. and Boyan, B.D. (1992) 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ regulation of arachidonic acid turnover in chondrocyte cultures is cell maturation-specific and may involve direct effects of phospholipase A_2. Biochim. Biophys. Acta., **1136**:45-51.

Swain, L.D., Schwartz, Z., Caulfield, K., Brooks, B. and Boyan, B.D. (1993) Nongenomic regulation of chondrocyte membrane fluidity by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is dependent on cell maturation. Bone, **14**:609-617.

Takai, Y., Kishimoto, Y., Iwasa, Y., Kawahara, T. and Nishizuka, Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipid. J.Biol.Chem., **254**:3692-3695.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun., **91**:1218-1224.

Tang, W., Ziboh, V.A., Isseroff, R.R. and Martinez, D. (1987) Novel regulatory actions of 1-alpha 1,25-dihydroxyvitamin D_3 on the metabolism of polyphosphoinositides in murine epidermal keratinocytes. J. Cell. Physiol., **132**:131-136.

van Leeuwen, J., Birkenhager, J.C., van den Bemd, G., Buurman, C.J., Staal, A., Bos, M.P. and Pols, H. (1992) Evidence for the functional involvement of protein kinase C in the action of 1,25-dihydroxyvitamin D_3 in bone. J. Biol. Chem., **267**:12562-12569.

Wahl, S.M., Allen, J.B., Wong, H. and Dasch, J. (1993) Reversal of acute and chronic synovial inflammation by anti-TGF-beta. J. Exp. Med., **177**:225-230.

Wali, R.K., Baum, C.L., Sitrin, M.D. and Brasitus, T.A. (1990) 1,25-$(OH)_2D_3$ vitamin D_3 stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. J. Clin. Invest., **85**:1296-1303.

Wrenn, R.W., Raeuber, C.L., Herman, L.E., Walton, W.J. and Rosenquist, T.H. (1993) Transforming growth factor-beta: signal transduction via protein kinase C in cultured embryonic vascular smooth muscle cells. In Vitro Cell. Dev. Biol., **29A**:73-78.

Yada, Y., Ozeki, T., Meguro, S., Mori, S. and Nozawa, Y. (1989) Signal transduction in the onset of terminal keratinocyte differentiation induced by 1-alpha, 25-dihydroxyvitamin D_3: role of protein kinase C translocation. Biochem. Biophys. Res. Commun., **163**:1517-1522.

VITA

Scott Arthur Mackey was born in Solon, Ohio on August 8, 1962 to Dr. Harold and LaVerne Mackey. Dr. Mackey attended Case Western Reserve University in Cleveland, Ohio and graduated in 1984 summa cum laude with a Bachelor of Arts degree in chemistry. During his undergraduate years, he was elected to membership in the Phi Beta Kappa national honor society. Dr. Mackey received his Doctor of Dental Surgery degree from the University of Michigan in 1988. During dental school Dr. Mackey received the Wilfrid T. Dempster Award for excellence in anatomy, the Dr. William S. Kramer Award of Excellence from the Omicron Kappa Upsilon Dental Society, and was elected to membership in the Omicron Kappa Upsilon National Honor Society. After dental school, Dr. Mackey was commissioned as an officer in the United States Air Force and attended a general practice residency program at Offutt Air Force Base, Nebraska. After being stationed for three years at Royal Air Force Upper Heyford, England, Dr. Mackey was selected in June, 1992 for graduate training in periodontics at Wilford Hall Medical Center, Lackland Air Force Base, Texas and the University of Texas Health Science Center at San Antonio. Dr. Mackey was married to Sharon L. Hill on 24 May 1986. They currently have three children Joshua Alexander, born 11 July 1988, Jessica Ann, born 29 November 1989, and Jennie Irene, born on 11 February 1992.