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REGULATION OF PROTEIN KINASE C EXPRESSION AND ACTIVITY BY 1,25-DIHYDROXYVITAMIN D_3 IN RAT OSTEOBLAST CELLS (ROS 17/2.8) AND ITS FUNCTIONAL IMPLICATION IN THE REGULATION OF OSTEOCALCIN GENE EXPRESSION

By

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REGULATION OF PROTEIN KINASE C EXPRESSION AND ACTIVITY BY 1,25-DIHYDROXYVITAMIN D_3 IN RAT OSTEOBLAST CELLS (ROS 17/2.8) AND ITS FUNCTIONAL IMPLICATION IN THE REGULATION OF OSTEOCALCIN GENE EXPRESSION

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Under the supervision of Professor William S. Mellon at the University of Wisconsin-Madison

The effect of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on protein kinase C (PKC) expression and activity in ROS 17/2.8 cells and the involvement of PKC signaling pathway in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression was investigated. We determined expression of PKCα, βI, ζ and ε in ROS 17/2.8 cells. 20 nM 1,25-$(OH)_2D_3$ increased PKCα, but not PKCβI, ζ or ε, levels in the nuclei in a time-dependent manner, as determined by immunoblotting. Whereas, the association of any of the expressed PKC isoforms with the plasma membrane was not altered with 1,25-$(OH)_2D_3$ treatment. Treatment for 15 minutes, 30 minutes, 1 hour and 24 hours increased PKCα nuclear levels by 2.3-2.6-fold. PKCα nuclear levels were also increased at 30 minutes, 1 hour and 24 hours treatment with 2 nM 1,25-$(OH)_2D_3$ by 1.8-2.7-fold. Moreover, PKCα nuclear levels were increased with doses of 1,25-$(OH)_2D_3$ as low as 0.05 nM. In contrast, two other vitamin D_3 metabolites, 24(R)-25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3, failed to increase PKCα nuclear levels.

To further substantiate the effect of 1,25-$(OH)_2D_3$ on PKCα levels, we determined PKCα subcellular localization after 20 nM 1,25-$(OH)_2D_3$ treatment with epi-fluorescence and confocal microscopy. In accord with the immunoblotting data, PKCα fluorescence in the nuclear compartment was increased with 1,25-$(OH)_2D_3$ treatment in a time-dependent manner. The percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence was higher at 30 minutes, 1 hour and 24 hours, but not at 6 or 12 hours. Moreover, the 1,25-$(OH)_2D_3$-induced PKCα nuclear levels was accompanied by a concomitant decrease in the cytosolic levels, indicating translocation. We corroborated by optical sectioning of immunostained ROS 17/2.8 cells that the PKCα signal detected in the nuclei by epi-fluorescence truly reflected nuclear PKCα.

To examine possible mechanisms involved in the 1,25-$(OH)_2D_3$-increased PKCα nuclear levels, we investigated the effect of 1,25-$(OH)_2D_3$ on PKCα mRNA levels by RT-PCR. 1,25-$(OH)_2D_3$ did not significantly alter PKCα mRNA levels in ROS 17/2.8 cells. To further substantiate that 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by inducing translocation of PKCα from the cytosol to this compartment, the effect of transcriptional inhibition on nuclear PKCα protein levels was studied. Inhibition of transcription with actinomycin-D did not alter the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels.

The PKC signaling pathway has been implicated in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin synthesis. However, no direct evidence for a role of a particular PKC isoform(s) in the regulation of osteocalcin gene expression has been presented. We determined the role of PKCα on steady state and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels in ROS 17/2.8 cells

since 1,25-$(OH)_2D_3$ specifically regulated this isoform. Overexpression of PKCα did not alter osteocalcin mRNA steady state levels. On the other hand, PKCα overexpression inhibited the 1,25-$(OH)_2D_3$-induced osteocalcin mRNA expression by over 75%.

06/25/01

Date

William S. Mellon

Professor

To my beautiful and lovable niece Leira Delis Rivera Santiago.

To the thousands of immigrants that come to this country searching for the "American Dream". I dedicate to them this thesis since with their hard work and dedication they contribute to the economy and prosperity of our country. Their contribution to this country has always been underestimated.

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Chapter I

INTRODUCTION

PREFACE

Vitamin D plays a major role in regulating mineral homeostasis in bone. This hormone may also be involved in bone resorption and bone formation. However, the signaling mechanism involved in vitamin D actions in bone remain elusive. Over the last decade, several reports have implicated protein kinases, particularly protein kinase C (PKC), in the actions of vitamin D. The exact role played by this protein kinase family in vitamin D-mediated actions is not yet well defined.

In this chapter vitamin D mediated regulation of gene expression is described. In Part I a special emphasis is given to the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression and the role of protein kinase C (PKC) signaling pathway in osteocalcin regulation. Part II provides an overview of the PKC signaling cascade and its nuclear mediated actions. The regulation of PKC gene expression by 1,25-$(OH)_2D_3$ is also discussed.

Part I: Vitamin D-Mediated Regulation of Gene Expression and Nongenomic Actions

Discovery

In 1919 Mellanby showed that the disease rickets in dogs could be cured with cod liver oil included in their diets (Mellanby, 1919). The same year Huldschinsky showed that the disease rickets in humans could be cured by exposing children to sunlight (Huldschinsky, 1919). Three years later, McCollum, at the University of Wisconsin-Madison, demonstrated that a factor different than vitamin A and present in cod liver oil cured rickets and played a critical role in its prevention (McCollum et al., 1922). He called the factor vitamin D. Steenbock, also at the University of Wisconsin-Madison, then reported that an inactive lipid was converted to vitamin D by ultraviolet light (Steenbock and Black, 1924, Steenbock and Black, 1925). Vitamin D_3 was definitively identified and its structure was elucidated in the 1930's (Askew et al., 1931, Windaus et al., 1936).

Vitamin D metabolism, activation and actions

It was first thought that vitamin D was the active substance and was not further converted to an active metabolite. It was then determined that vitamin D was first metabolized to an active form before mediating its effects (Lund and DeLuca, 1966). The first active metabolite of vitamin D, 25-hydroxyvitamin D_3 (25-OH-D_3), was isolated in 1968 (Blunt et al., 1968). 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the main active metabolite, was isolated and characterized in the early 1970's (Holick et al., 1971).

7-dehydrocholesterol, the main source of vitamin D in humans, is converted to previtamin D_3 by ultraviolet light (Holick, 1996). Previtamin D_3 undergoes an internal isomerization of its three double bonds to form 5,6-*cis*-triene and is transformed to vitamin D_3. Vitamin D_3 is transported to the liver where it is hydroxylated at C-25 resulting in the formation of 25-hydroxyvitamin D_3 [25-OH-D_3] by 25-hydroxylase. 25-OH-D_3 in then transported to the kidney where it is metabolized by 1α-hydroxylase in the proximal tubule to form 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. 1,25-$(OH)_2D_3$ is the active form of vitamin D. 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] is also produced in the kidney by the enzyme 24-hydroxylase. This metabolite may be involved in cellular functions but the data is still controversial (Darwish and DeLuca, 1993, Norman and Collins, 1996).

The production of 1,25-$(OH)_2D_3$ is controlled by the levels of calcium and phosphorous in the circulation (Darwish and DeLuca, 1993). Low levels of serum calcium stimulate the release of the parathyroid hormone (PTH) from the parathyroid gland. PTH then induces the production of 1,25-$(OH)_2D_3$ by stimulating 1α-hydroxylase activity (Garabedian et al., 1972). Low levels of phosphorous in the circulation affect 1,25-$(OH)_2D_3$ production by inducing 1α-hydroxylase and repressing 24-hydroxylase activity. 1,25-$(OH)_2D_3$ also regulates its own production by inhibiting the 1α-hydroxylase enzyme. In addition to PTH, phosphorous, calcium and 1,25-$(OH)_2D_3$, other factors including sex hormones and growth factors, regulate 1,25-$(OH)_2D_3$ production (Tanaka et al., 1976, Spanos et al., 1976, Christakos, 1996).

Once produced, 1,25-$(OH)_2D_3$ stimulates the intestinal absorption of calcium and phosphorous from the diet. It acts in concert with the parathyroid hormone in the kidney to increase calcium absorption if sufficient calcium levels are not obtained. 1,25-$(OH)_2D_3$ also mobilizes calcium from bone to the circulation, if needed. The saturating levels of these two ions sustain neuromuscular activity and bone mineralization.

1,25-$(OH)_2D_3$ also affects other cellular targets (Christakos, 1996, Holick, 1996). It inhibits proliferation and stimulates the differentiation of several cell lines including leukemia cells (Hosomi et al., 1983, Shavit et al., 1983, Lemire et al., 1984, Rigby et al., 1984, Lee et al., 1987, Suda, 1989). Inhibition of proliferation of several malignant cells such as colon and prostate cancer cells has also been reported (Christakos, 1996). 1,25-$(OH)_2D_3$ has been shown to suppress T cell proliferation and to regulate the expression of several cytokines (interleukins 1, 2, 3 and 6, and γ-interferon) (Manolagas et al., 1994).

1,25-$(OH)_2D_3$-mediated regulation of gene expression

1,25-$(OH)_2D_3$ mediates it action by acting through intracellular receptors and regulating the expression of genes in target tissues such as the intestine, kidney and bone. The first evidence of the existence of a nuclear vitamin D receptor (VDR) was reported by Brumbaugh and Haussler (Brumbaugh and Haussler, 1975). With the help of monoclonal antibodies, the molecular cloning of VDR cDNA for chicken (McDonnell et al., 1987), rat (Burmester et al., 1988), and human (Baker et al., 1988) was established.

VDR is part of the nuclear hormone receptor superfamily. In mammals a single VDR has been identified in all target tissues. Two forms exist in avian tissue. The VDR is composed of a DNA binding domain at the amino terminus and a ligand binding domain at the carboxy terminus. These two regions are linked by a hinge region. The DNA binding domain contains two zinc-fingers that bind to vitamin D response elements (VDRE) in the target genes. This VDRE consists of two repeats separated by three nucleotides. The mechanism of action includes binding of 1,25-$(OH)_2D_3$ to its intracellular receptor, followed by binding of the hormone receptor complex to hormone response elements on DNA with the concomitant activation or repression of transcription. The VDR binds to VDRE in complex with a nuclear accessory factor. It has been shown that this accessory factor is the retinoic acid receptor (RXR) (Munder et al., 1995).

Phosphorylation of VDR is an early event in the mechanism of 1,25-$(OH)_2D_3$ action. Brown and DeLuca showed phosphorylation of VDR to be localized at a 23-KDa segment of the carboxy terminus domain in cultured chick duodena (Brown and DeLuca, 1991). In their studies phosphorylation involved serine residues. Jurutka et al. proposed that in the human VDR phosphorylation occurs at Ser208 and is mediated by casein kinase-II *in vitro* (Jurutka et al., 1993). On the contrary, Hsieh and coworkers proposed that human VDR is phosphorylated by PKCβ at SER51 both *in vitro* and *in vivo* (Hsieh et al., 1991, Hsieh et al., 1993). Hsieh's results contradict both Brown and Deluca's, and Jurutka's reports since Ser51 lies outside the 23KDa fragment. Darwish and coworkers propose that protein kinase A (PKA) is involved in VDR

phosphorylation (Darwish and DeLuca, 1993). Moreover, they demonstrated that phosphorylation is required for VDR-mediated transcriptional activity.

VDR expression has been reported in over 35 tissues and more that 50 genes are known to be regulated by 1,25-$(OH)_2D_3$ (Darwish and DeLuca, 1993, Norman and Collins, 1996, Christakos, 1996). Some examples are presented therein. In the intestine and kidney, 1,25-$(OH)_2D_3$ increases the synthesis of calcium binding proteins known as calbindins (calbindin 9-KDa and calbindin 28-KDa). A VDRE has been identified in the promoter region of calbindin 9K (Darwish and DeLuca, 1992). Another example is the regulation of 24-hydroxylase gene expression. 24-hydroxylase is the first gene reported to be controlled by two independent VDREs. It has been postulated that the proximal VDRE is more responsive to 1,25-$(OH)_2D_3$ than the distal one (Christakos, 1996). Osteocalcin, a protein produced by osteoblasts, is also up-regulated by 1,25-$(OH)_2D_3$. The regulation and function of this protein is discussed later.

On the other hand, 1,25-$(OH)_2D_3$ has been shown to down-regulate the expression of several genes. Our laboratory and others showed that 1,25-$(OH)_2D_3$ decreased c-myc mRNA levels in HL-60 cells (Simpson et al., 1987, Mangasarian and Mellon, 1993). It was shown by our laboratory that the 1,25-$(OH)_2D_3$-mediated decrease in c-myc expression occurs at the posttranscriptional level. Another gene known to be down-regulated by 1,25-$(OH)_2D_3$ is the parathyroid hormone. A negative VDRE is contained in the promoter region of the human parathyroid hormone gene and only a single motif has been identified (Demay et al., 1992).

Finally another gene down-regulated by 1,25-$(OH)_2D_3$ is interleukin-2 (IL-2). Alroy et al reported that the VDR/RXR complex block the transcription factors NFATp and jun/fos to bind to the IL-2 promoter (Alroy et al., 1995). This results in inhibition of IL-2 transcription.

Vitamin D nongenomic actions

Several reports suggest that some of the 1,25-$(OH)_2D_3$ cellular effects are mediated by a mechanism independent of gene regulation (Revelli et al., 1998). First, these effects occur too fast to be concurrent with gene expression. Second, they occur in cell lines that do not express the VDR. Moreover, they are not blocked by transcriptional and translational inhibitors. These so called "non-genomic" effects include: rapid Ca^{2+} translocation in perfused chick duodenal loops (Nemere et al., 1984, Nemere and Norman, 1987), increases in intracellular Ca^{2+} levels in rat hepatocytes and in chick skeletal muscle cells (Baran and Milne, 1986, Capiati et al., 2000), and regulation of phospholipids metabolism in chick myoblasts and rat hepatocytes (Morelli et al., 1993, de Boland et al., 1994, Baran et al., 1989). 1,25-$(OH)_2D_3$ has also been shown to modulate PKC activity in several cell lines. In Madin Darby bovine kidney (MDBK) cells, 1,25-$(OH)_2D_3$ increases PKCα association with the plasma membrane and induces translocation of PKCβ to the nuclei (Simboli-Campbell et al., 1994). In human promyelocytic leukemia NB4 cells, 1,25-$(OH)_2D_3$ induces translocation of PKCδ to the nuclei, and PKCα to the nuclei and to a particulate fraction (Berry et al., 1996). In human colon cancer-derived Caco-2 cells, 1,25-$(OH)_2D_3$ activates PKCα and this isoform in turn limit the 1,25-$(OH)_2D_3$-stimulated rise in intracellular Ca^{+2} (Bissonnette et al., 1994).

Non-genomic effects of 1,25-$(OH)_2D_3$ have been reported in bone cells. 1,25-$(OH)_2D_3$ has been shown to increase intracellular Ca^{2+} concentration in confluent mouse osteoblasts and in rat-derived osteosarcoma cells (Lieberherr, 1987, Civitelli et al., 1990, Baran et al., 1991), to modulate dihydropyridine-sensitive Ca^{2+} currents in ROS 17/2.8 cells (Caffrey and Farach-Carson, 1989), and to alter phospholipids metabolism in confluent mouse osteoblast and in ROS 17/2.8 cells (Grosse et al., 1993, Sorensen and Baran, 1995).

1,25-$(OH)_2D_3$ actions in bone and osteocalcin regulation

The mechanism by which 1,25-$(OH)_2D_3$ regulates mineral homeostasis is still not well understood. Bone turnover is mostly regulated by osteoblasts and osteoclasts. While osteoclasts are involved in bone resorption, the osteoblast main function is to synthesize new bone (Marks Jr. and Hermey, 1996). To maintain an adequate mineral balance as well as an appropriate bone mass, this process has to be strictly regulated. 1,25-$(OH)_2D_3$ has been shown to enhance bone resorption in vitro (i.e. activate osteoclasts) (McSheehy and Chambers, 1987), but VDR expression has not been reported in osteoclasts. It has been proposed that 1,25-$(OH)_2D_3$ induces osteoblasts to secrete a soluble factor(s) that increases osteoclastic bone resorptive activity. Osteocalcin, the major noncollagenous protein present in the extracellular matrix and produced by osteoblasts, has been proposed as a possible candidate. Several reports support this idea. First, a decreased synthesis of osteocalcin has been correlated with poorly resorbed bone (Lian et al., 1984, DeFranco et al., 1991). Finally, osteocalcin has been shown to be involved in recruiting

and stimulating the differentiation of osteoclast progenitors (Glowacki and Lian, 1987, Liggett et al., 1994)

Evidence against the role of osteocalcin in bone resorption has also been presented. Vitamin K-deficient rats, whose bone osteocalcin levels were 2% of normal, showed no abnormalities in bone size and mineralization (Price and Williamson, 1981). Ducy et al. generated osteocalcin-deficient mice. These mice developed a phenotype characterized by a higher bone mass that normal mice (Ducy et al., 1996). However, the "loss-of-function" of osteocalcin did not appear to affect either bone resorption or bone mineralization. They suggest that the function of osteocalcin is to limit bone formation without lessening bone resorption. Recently, Boskey et al. reported a crystal maturation defect in osteocalcin-deficent mice as detected by fourier transform infrared microspectroscopic anaysis (Boskey et al., 1998), suggesting that osteocalcin is needed to stimulate bone mineral maturation.

Osteocalcin is the most abundant noncollagenous protein in bone. This protein is expressed by osteoblasts and odontoblasts. It is synthesize in a pre-propeptide as a 10 KDa, but is secreted as a 5.6 KDa peptide (Lian et al., 1999). Osteocalcin contains three γ-glutamic acid (GLA) residues that are formed after processing in a vitamin K-dependent reaction. These residues are important for interaction with divalent cations and for binding to the hydroxyapatite component of the extracellular matrix.

The regulation of osteocalcin has been used as a model to study the actions of 1,25-$(OH)_2D_3$ in bone regardless of the function(s) it may mediate. 1,25-$(OH)_2D_3$ has been shown to increase osteocalcin gene expression at the transcriptional level (Demay et al., 1989, Breen et al., 1994, Mosavin and Mellon, 1996). Osteocalcin genes have been cloned and characterized from several species including rat and human (Yoon et al., 1988, Ducy and Karsenty, 1996). The osteocalcin promoter has been well characterized (Lian et al., 1999). The 5' flanking region of the rat and human osteocalcin gene contains sequences that confer responsiveness to 1,25-$(OH)_2D_3$ (Demay et al., 1989, Demay et al., 1990, Breen et al., 1994, Kerner et al., 1989). At least two regions in the rat osteocalcin promoter contribute to 1,25-$(OH)_2D_3$ responsiveness. However, Demay and coworkers demonstrated by mutational analysis that the proximal region was able to confer hormonal response to either the native osteocalcin promoter or to a heterologous viral promoter (Demay et al., 1990). This sequence lies 458 base pairs up-stream from the transcription start site and contain three copies of a short sequence that are homologous to "half-sites" of other steroid response elements. The vitamin D response elements present in the rat and human osteocalcin gene are GGGTGA atg AGGACA (-455/-441) and GGGTGA acg GGGGCA (-499/-485), respectively (Christakos, 1996). Our laboratory demonstrated that 1,25-$(OH)_2D_3$ also regulates osteocalcin expression at the posttranscriptional level (Mosavin and Mellon, 1996). However, the molecular mechanism(s) by which 1,25-$(OH)_2D_3$ regulates osteocalcin transcription and message stabilization are still not well understood.

The rat osteocalcin promoter contains three AP-1 sites. One of these sites is contained in the VDRE. This AP-1 element is necessary to support responsiveness to 1,25-$(OH)_2D_3$ (Aslam et al., 1999). However, binding of transcritpion factors such as c-fos/c-jun to this site blocks binding of the VDR to the VDRE (Owen et al., 1990). The rat osteocalcin promoter also contains two glucocorticoid response elements (GRE), a transforming growth factor (TGF-β) response element (TGRE), and a cyclic AMP response motif (cAMP) (Lian et al., 1999). These regions may mediate the regulation of osteocalcin by a variety of factors including parathyroid hormone (Yu and Chandrasekhar, 1997), glucocorticoids (Shalhoub et al., 1998), growth factors (Noda, 1989), and cAMP (Evans et al., 1989, Boguslawski et al., 2000).

Evidence for the functional involvement of PKC in the actions of 1,25-$(OH)_2D_3$ in bone with respect to osteocalcin regulation has been reported. Our laboratory and others reported that treatment of ROS 17/2.8 cells with several PKC inhibitors blocked 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis (van Leeuwen et al., 1992, Mosavin, 1996). In another study, the PKC inhibitor staurosporine was shown to inhibit the 1,25-$(OH)_2D_3$-mediated induction of osteocalcin gene transcription (Desai et al., 1995). Buguslawski and coworkers also reported that osteocalcin transcription involves a PKC-dependent pathway (Boguslawski et al., 2000). Our laboratory demonstrated that bisindolylmaleimide (a potent PKC inhibitor) blocked the 1,25-$(OH)_2D_3$-mediated stabilization of the osteocalcin message (Mosavin, 1996).

In summary, osteocalcin functions are still not fully defined and the molecular mechanism(s) involved in its regulation by 1,25-$(OH)_2D_3$ are still not well characterized. The PKC signaling pathway has been implicated in the actions of 1,25-$(OH)_2D_3$ in bone, particularly in the regulation of osteocalcin gene expression.

Part II. Protein Kinase C Signaling Pathway

Discovery and characterization

During the course of their studies on cyclic GMP- and cyclic AMP-dependent protein kinases Nishizuka's group discovered another protein kinase (Takai et al., 1977). They observed that this enzyme was independent of cyclic nucleotides, showed kinetic properties partly similar to those of cyclic GMP-dependent protein kinase, and was distinguishable from both the catalytic subunit of cyclic AMP-dependent protein kinase and the catalytic fragment of cyclic GMP-protein kinase. This kinase that was partially purified from bovine cerebellum phosphorylated histone and protamine. In an accompanying paper the properties of the proenzyme and enzyme isolated from the soluble fraction of rat brain were reported (Inoue et al., 1977). The active enzyme was produced from its proenzyme by a proteolytic reaction catalyzed by a calcium-dependent protease. It was also detected in various tissues and organs in rats including lung, liver, kidney, cerebellum, heart, skeletal muscle and adipose tissue. They named the active enzyme PKM.

A role of this kinase as a mediator of intracellular signaling was later established from reports of Nishizuka's lab. First, they reported that the proenzyme was active without proteolysis when a membrane-associated factor together with calcium was present in the reaction (Takai et al., 1979a). This factor that was almost exclusively localized in membranes was extracted and recovered in the phospholipid fraction. Moreover, they showed that pure phosphatidylinositol,

phosphatidylserine, phosphatidic acid and disphosphatidylglycerol could replace the factor. They called the enzyme PKC (Ca^{2+}-dependent protein kinase). Later, the same laboratory reported that the enzyme was activated by diacylglycerol (DAG), suggesting that membrane metabolism appeared to be pivotal for activation of PKC (Takai et al., 1979b, Kishimoto et al., 1980). A real interest in PKC was generated with the announcement that this enzyme was activated by tumor-promoting phorbol esters (Castagna et al., 1982). They reported that as DAG, TPA increased the affinity of the enzyme for Ca^{2+} and phospholipids, thus enhancing PKC activation. Later, it was shown that phorbol ester treatment decreased PKC cytosolic levels in EL4 thymoma cells (Kraft et al., 1982). This decrease in cytosolic PKC was later correlated to an increase in the plasma levels of the enzyme by phorbol esters (Kraft and Anderson, 1983), suggesting translocation of the enzyme from the cytosol to the membrane upon activation. A model for Ca^{2+}- and phorbol ester-mediated PKC translocation was definitively presented by Wolf and coworkers (Wolf et al., 1985). Finally, the complete primary structure of PKC was reported by Parker et al. in 1986 (Parker et al., 1986).

PKC structure, regulation and enzymological properties

Protein Kinase C is a family of serine/threonine kinases that consist of at least 12 isoenzymes. These isoforms differ in tissue expression, intracellular localization and mode of activation (Dekker and Parker, 1994, Nishizuka, 1995). Based on their structure they are divided into three major subfamilies. The first group, the classical or conventional PKC, consists of α, βI, βII and γ isoforms. These isoforms are activated by Ca^{2+}, diacylglycerol (DAG), phorbol esters,

phosphatidylserine (PS) and *cis*-unsaturated fatty acids. The novel isoforms [δ, ε, η(L), θ and μ] are regulated by DAG, phorbol esters and PS, but not by Ca^{2+}. The last subfamily, the atypical PKCs, consists of ζ, ι and λ isoforms. They are not activated by Ca^{2+}, DAG or phorbol esters. Evidence suggests that PKCζ is activated by PS, free fatty acids and phosphatidylinositol 3,4,5-trisphosphate (Nishizuka, 1995, Nakanishi et al., 1993).

PKC is composed of a regulatory domain and a catalytic domain. These domains contain regions common (C) between the isoforms and variable (V) regions unique to each isoform. The variable regions may mediate functions unique to the individual isoforms, but this has not been yet proved. The C1 region contains two repeats of a cysteine-rich motif in the classical and novel PKCs, but only one in the atypicals. This region mediates DAG, phorbol ester and lipids (i.e. PS) binding in the classical and novel PKCs. Absence of one cysteine-rich motif in the atypical isoforms may explain why they are not regulated by either DAG or phorbol esters. C1 ligands markedly reduce the concentration of Ca^{2+} required for the phosphatidylserine-dependent activation of PKC. The structure of the second activator-binding domain of PKCδ complexed with phorbol-13-acetate has been determined by crystallography (Zhang et al., 1995). Rather than inducing a conformation change, phorbol ester binding forms a contiguous hydrophobic surface covering one-third of the domain, inducing PKC insertion into the membrane. The C1 region also contains a pseudosubstrate sequence that maintains the enzyme in its inactive state (House and Kemp, 1987).

The conventional PKCs contain a C2 region that mediates Ca^{2+} binding and is also involved in PKC translocation to membranes. Ca^{2+} increases the affinity of PKC for negatively charged phospholipids. A "C2-like" domain is present in novel PKCs, but this region does not contain the functional groups that bind Ca^{2+}.

The regulatory domain consists of an ATP binding site (C3) and the substrate binding site (C4). PKC uses MgATP as substrate to phosphorylate serine and threonine residues in basic sequences on target proteins. PKC displays less specificity than PKA (Kennelly and Krebs, 1991, Newton, 1995). First, unlike PKA, no requirement for positive charge at specific positions is apparent. Second, PKC displays lower stereospecificity than PKA, phosphorylating both D- and L-stereoisomers of α configurational isomers on a number of alcohols (Kwon et al., 1994). PKC autophosphorylates in vitro by three functional distinct phosphorylation at the N terminus, hinge, and C terminus (Newton, 1995). These autophosphorylations provide PKC catalytic activity and localizes the enzyme to the cytosol.

PKC signaling pathway and nuclear mediated actions

PKC are mostly present in the cytoplasm in an inactive state. Ligand-activation of membrane receptors, including seven-spanning membrane receptors, stimulates phospholipase C activity (Nishizuka, 1995). PLC then hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in the generation of DAG and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to IP_3 receptors located in the endoplasmic reticulum resulting in an increase in $[Ca^{2+}]_i$. This generation of DAG

last seconds to a few minutes. A second phase of DAG generation, that persists for minutes to hours, is mediated by the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) and/or phospholipase A_2 (PLA_2). Generation of DAG and Ca^{2+} results in translocation of PKC to the plasma membrane. However, in the last decade several studies have demonstrated that PKC translocates to other cellular compartments including golgi (Goodnight et al., 1995, Prestle et al., 1996, Westermann et al., 1996), cytoskeleton (Kiley and Parker, 1995, Goodnight et al., 1995), perinucleus (Disatnik et al., 1994, Kiley and Parker, 1995), and endoplasmic reticulum (Chida et al., 1994, Goodnight et al., 1995).

PKC has been implicated in cellular responses to a vast array of agonists such as growth factors and hormones (Dekker and Parker, 1994, Nishizuka, 1995). Besides PKC's role in signaling transduction events, emerging data suggests a role of PKC in "long-term" cellular functions such as proliferation and differentiation (Solomon et al., 1991, Brooks et al., 1993, Dekker and Parker, 1994, Gamard et al., 1994, Nishizuka, 1995, Simpson et al., 1998, Capiati et al., 1999, Capiati et al., 2000). Many of the physiological functions mediated by PKC involve nuclear events. For instance, PKC has been shown to phosphorylate DNA polymerase α and β (*in vitro*), RNA Polymerase II (*in vitro*), VDR (*in vitro* /*in vivo*), and CREB (*in vitro*) (Buchner, 1995, Hsieh et al., 1991, Hsieh et al., 1993). Moreover, PKC signaling pathway has been implicated in the regulation of expression of several genes (Auwerx et al., 1989, Perrone-Bizzozero et al., 1993, Chen et al., 1994, Pang et al., 1996, Li et al., 1998).

How PKC once activated brings the transduction signal to the nuclei is a subject of continuous investigation. Several possibilities are discussed therein. First, PKC may phosphorylate another kinase present in the cytoplasm and activation of this cascade triggers a nuclear-directed cascade. A well-characterized system is the mitogen-activated protein kinase (MAPK) cascade (Buchner, 1995). PKC has been shown to phosphorylate raf-1 both *in vitro* and *in vivo* (Kolch et al., 1993). This leads to phosphorylation of MAP kinase kinase followed by MAPK activation and translocation to the nucleus. Another possibility is that PKC itself translocates to the nuclei upon activation. Several reports have demonstrated PKC nuclear translocation by various growth factors and hormones including α-thrombin and platelet-derived growth factor (Dekker and Parker, 1994), retinoic acid (Zorn and Suaro, 1995, Gruber et al., 1995), and vitamin D_3 (Simboli-Campbell et al., 1994, Berry et al., 1996).

PKC may also translocate to the nuclei in an inactive state, followed by activation in the nuclei. The existence of a phosphoinositide cycle in the nucleus has been demonstrated in Swiss 3T3 fibroblasts cells (Divecha et al., 1991) and in IIC9 fibroblasts (Jarpe et al., 1994). In the first report, stimulation of Swiss 3T3 cells with IGF-1 leads to a rapid and transient decrease in nuclear phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate with the concomitant increase in nuclear DAG. On the other hand, Jarpe et al. reported that α-thrombin induces DAG generation from phosphatidylcholine. It appears that DAG can be produced in the nuclei envelope by phospholipid metabolism. Nuclear PKC may also be activated by nuclear Ca^{2+}. Several reports have suggested that the nucleus can regulate Ca^{2+} concentrations

independently form the cytosol (Nicotera et al., 1989, Hernández-Cruz et al., 1990, Nicotera et al., 1990, Malviya et al., 1990, Przywara et al., 1991, Birch et al., 1992, Himpens et al., 1992). However, these reports have been challenged (Al-Mohanna et al., 1994, Buchner, 1995). Nevertheless, these reports show that Ca^{2+} levels in the nuclei can reach physiological values.

Regulation of PKC gene expression

Regulation of PKC gene expression by hormones has been reported. In Chinese hamster ovary (CHO) cells treatment with insulin increased PKCα mRNA levels in a time- and concentration-dependent manner as detected by RT-PCR (Antoine et al., 1998). In melanoma cells, retinoic acid increased PKCα mRNA levels beginning at 4 hours with maximal induction (10-12-fold) at 48 hours (Rosenbaum and Niles, 1992). However, a modest increase in PKCα transcriptional rate (2-3-fold) was detected by nuclear run-on experiments, suggesting that retinoic acid regulates PKCα mRNA at the posttranscriptional level in this cell line. A retinoic acid response element in the promoter of the murine PKCα gene has been identified (Desai et al., 1999). 1,25-$(OH)_2D_3$ also regulates PKCα and PKCβ gene expression in HL-60 cells (Obeid et al., 1990, Solomon et al., 1991). Obeid and coworkers reported a 4-5-fold increase in PKCβ and PKCα steady state mRNA levels at 24-48 hours treatment with 1,25-$(OH)_2D_3$ (Obeid et al., 1990). Solomon et al. also reported increases in steady state PKCα, βI and βII mRNA with maximal induction at 48 hours (Solomon et al., 1991). However, while Obeid and coworkers reported increases in transcriptional rates for these isoforms after treatment with 1,25-$(OH)_2D_3$, Solomon and coworkers were not able to detect changes on the PKCα, βI and βII rates of transcription. The

apparent discrepancy may be due to the fact that to normalize the rate of transcription of PKCβs and PKCα genes different internal control genes were used between the studies. Nevertheless, 1,25-$(OH)_2D_3$ regulates PKC gene expression in HL-60 cells. Regulation of PKC gene expression by 1,25-$(OH)_2D_3$ in bone cells has not been reported

PKC actions in bone

A role for PKC in bone resorption has been proposed (Bos et al., 1992, Lee and Stern, 2000). Moreover, evidence for the functional involvement of PKC in the actions of 1,25-$(OH)_2D_3$ in bone in respect to osteocalcin regulation has also been reported (van Leeuwen et al., 1992, Mosavin, 1996, Desai et al., 1995, Boguslawski et al., 2000).

STATEMENT OF THE PROBLEM

Osteocalcin, the most abundant noncollagenous protein in the extracellular matrix, is proposed to play a role in bone resorption and mineralization. The regulation of this protein by 1,25-$(OH)_2D_3$ has been used as a model to study 1,25-$(OH)_2D_3$-mediated regulation of gene expression. 1,25-$(OH)_2D_3$ is known to regulate osteocalcin expression both at the transcriptional and posttranscriptional levels. However, the molecular mechanism by which 1,25-$(OH)_2D_3$ regulates the transcriptional activation and stabilization of osteocalcin mRNA are still not well understood. Several reports have implicated PKC signaling pathway in 1,25-$(OH)_2D_3$-mediated actions in bone, specifically in the regulation of osteocalcin gene expression. However, regulation of PKC expression and activity by 1,25-$(OH)_2D_3$ in bone cells has not been studied. The main focus of this thesis is to study the regulation of PKC expression and translocation by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells and to determine the role of PKC isoforms in 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression.

Chapter 2 examines the regulation of PKC expression and activity by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. Western blotting was used to determine the effect of 1,25-$(OH)_2D_3$ treatment on PKC isoforms subcellular localization. The effect of two other vitamin D_3 metabolites [24,25-$(OH)_2D_3$ and 25-OH-D_3] on PKCα activity was also determined.

Epi-fluorescence and confocal microscopy were used to study 1,25-$(OH)_2D_3$-mediated regulation of PKCα subcellular localization. The results of these experiments are presented in Chapter 3.

In Chapter 4, the role of PKCα in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression was studied. PKCα was overexpressed in ROS 17/2.8 cells and the effect of the PKCα overexpression on osteocalcin steady state and 1,25-$(OH)_2D_3$-induced mRNA levels was determined.

Chapter 5 summarizes the results obtained in our studies. The possible mechanisms involved in the 1,25-$(OH)_2D_3$-induced subcellular translocation of PKCα and the role of this isoform in 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin expression are discussed.

Finally, in the Appendix data concerning the effect of 1,25-$(OH)_2D_3$ on PKCα gene expression as studied by RT-PCR is presented. The effect of transcriptional inhibition on 1,25-$(OH)_2D_3$-mediated PKCα nuclear translocation was also determined.

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Chapter II

1,25-Dihydroxyvitamin D_3 specifically translocates protein kinase Cα to the nuclei in rat osteoblast cells (ROS 17/2.8)

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ABSTRACT

We have investigated PKC regulation by 1,25-$(OH)_2D_3$ in the rat osteosarcoma cell line ROS 17/2.8 since previous reports have implicated PKC in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996 #637). In this chapter we report that 1,25-$(OH)_2D_3$ increased PKCα, but not PKCβI, ε or ζ, levels in the nuclear fraction in a time-dependent manner. Whereas, 1,25-$(OH)_2D_3$ did not alter the association of any of the expressed PKC isoenzymes with the plasma membrane. Treatment with 20 nM 1,25-$(OH)_2D_3$ for 15 minutes, 30 minutes, 1 hour and 24 hours increased PKCα levels in the nuclear fraction by 2.3- to 2.6-fold. Moreover, PKCα nuclear levels were also increased with doses of 1,25-$(OH)_2D_3$ as low as 0.2 and 0.05 nM. Whereas, 25-OH-D_3 and 24(R),25-$(OH)_2D_3$, two vitamin D_3 metabolites, in concentrations as high as 250 and 1000 nM, respectively, failed to increasing PKCα levels in the nuclei.

INTRODUCTION

Over the last few years several studies have implicated the involvement of several kinases in the actions of 1,25-$(OH)_2D_3$. Up-regulation, down-regulation and phosphorylation of the vitamin D receptor by PKA and PKC have been reported (Krishnan and Feldman, 1991, Darwish et al., 1993, Darwish and DeLuca, 1993, van Leeuwen et al., 1990, Hsieh et al., 1993). PKC has also been implicated in the actions of 1,25-$(OH)_2D_3$ in several cell types (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 1999, Simpson et al., 1998).

Protein Kinase C is a family of protein kinases that consist of at least 12 isoforms. They differ in tissue expression, intracellular localization and in enzymological properties (Dekker and Parker, 1994, Nishizuka, 1995). These enzymes are involved in cellular responses to a vast array of agonists such as neurotransmitters, growth factors and hormones. Besides PKC's role in signal transduction events, emerging data suggest a role in "long-term" cellular functions such as proliferation and differentiation (Solomon et al., 1991, Brooks et al., 1993, Dekker and Parker, 1994, Gamard et al., 1994, Nishizuka, 1995, Simpson et al., 1998, Capiati et al., 1999, Capiati et al., 2000).

Several studies have implicated a role for PKC in the action of 1,25-$(OH)_2D_3$ in kidney, leukemia, cancer-derived and skeletal muscle cells. In Madin Darby bovine kidney (MDBK) cells, treatment with 1,25-$(OH)_2D_3$ increased plasma membrane association of PKCα and induced translocation of PKCβ to the nuclei (Simboli-Campbell et al., 1994). In human promyelocytic leukemia NB4 cells, 1,25-$(OH)_2D_3$ induced translocation of PKCδ to the nuclei,

and PKCα to the nuclei and to a particulate fraction (Berry et al., 1996). In human colon cancer-derived Caco-2 cells, 1,25-$(OH)_2D_3$ activates PKCα and this isoform in turn limits the 1,25-$(OH)_2D_3$-stimulated rise in intracellular Ca^{+2} (Bissonnette et al., 1994). In chick myoblasts the early 1,25-$(OH)_2D_3$-mediated stimulation of myoblast proliferation correlated with an increase in PKCα expression whereas decreased PKCα levels is observed during the subsequent 1,25-$(OH)_2D_3$–induction of myoblast differentiation (Capiati et al., 1999). PKC (specifically the α isoform) has also been implicated in the 1,25-$(OH)_2D_3$ regulation of intracellular Ca^{+2} during development of skeletal muscle cells (Capiati et al., 2000).

Previous studies implicated PKC as a component of the signaling pathway of 1,25-$(OH)_2D_3$ in bone, particularly in the regulation of the osteocalcin gene. It has been shown by our laboratory and others that treatment of ROS 17/2.8 cells with PKC inhibitors reduced the 1,25-$(OH)_2D_3$-mediated induction of osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). Our laboratory also demonstrated that inhibition of PKC by bisindolylmaleimide blocked the 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA (Mosavin, 1996).

These results taken together provided the rationale for studying PKC expression and regulation by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. We determined the presence of PKCα, βI, ζ and ε in ROS 17/2.8 cells. In this chapter, we present evidence that 1,25-$(OH)_2D_3$ specifically regulates PKCα activity in a bone cell line. Our results indicate that 1,25-$(OH)_2D_3$ increases PKCα, but not PKCβI, ζ or ε, levels in the nuclei in a time-dependent manner. This 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels occurred early (15 minutes) after treatment, suggesting

translocation of PKCα from the cytosol to the nuclei. The 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels supports a role for this isoform in 1,25-$(OH)_2D_3$ nuclear actions.

MATERIAL AND METHODS

Materials 1,25-$(OH)_2D_3$, 24(R),25-$(OH)_2D_3$ and 25-OH-D_3 were kindly provided by Dr. Hector F. DeLuca (University of Wisconsin-Madison). Rabbit polyclonal IgG antibodies that recognize PKCα, βI, βII, γ, ζ and ε, and peroxidase-conjugated anti-rabbit IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, California). cPKCα, cPKCβI, cPKCβII, cPKCγ and nPKCε antibodies were raised against a peptide mapping at the carboxy terminus of their respective PKC isoform of human origin. nPKCζ antibody was raised against a peptide mapping at the carboxy terminus of nPKCζ of rat origin. All polyclonal antibodies react with their respective isoform of mouse, rat and human origin. Goat polyclonal antibody that recognizes Lamin B was obtained from Santa Cruz Biotechnology (Santa Cruz, California). Lamin B antibody was raised against a peptide mapping at the carboxy terminus of Lamin B of human origin. Lamin B antibody recognizes Lamin B of mouse, rat and human origin.

Cell culture ROS 17/2.8 cells were maintained in Ham's F-12 (1:5) culture medium supplemented with 5% (vol/vol) defined fetal bovine serum (Hyclone, Utah), penicillin (100 u/ml), and streptomycin (0.1 mg/ml). Cells were propagated continuously at 37^0C under a humidified 5% CO_2: 95% air mixture. For the experiments, cells were plated at a density of 1.0 x 10^4 cells/cm^2 on 55 cm^2 petri dishes and allowed to adhere overnight. When the cells reached 50-70% confluence they were treated with either ethanol (as control), 1,25-$(OH)_2D_3$ (0.05, 0.2, 2

and 20 nM) or PMA (100 nM) for a specified amount of time. Experiments were carried out on cells between the 5th and 30th passage.

Subcellular fractionation Cytosolic, plasma membrane and nuclear fractions were prepared as described before with some modifications (Suzuki et al., 1989, Delude et al., 1994, Kim et al., 1996). All the steps were performed at 4^0C.

Preparation of plasma membrane fraction The cells were washed and collected in a chilled TEDK buffer solution [10 mM Tris-HCl (pH 7.2), 0.3 M KCl, 1 mM EDTA, and 5 mM dithiothreitol (DTT)]. The cell suspension was homogenized in a type B Dounce homogenizer (Wheaton, USA) until greater than 95% of the cells were disrupted (40 strokes). The homogenized solution was then centrifuged at 500 x g for 10 minutes. The supernatant fluid was centrifuged at 27,000 x g for 30 minutes. The cell pellet was resuspended in 1.5 ml of 15% sucrose in TEDK and layered on a discontinuous sucrose density layer composed of 2.0 ml of 30% sucrose in TEDK layered on to 1.5 ml of 45% sucrose in TEDK. These TEDK solutions contained 1% protease inhibitor cocktail solution (Sigma, St. Louis, Missouri). The gradient was centrifuged at 76,000 x g for 3 hour. The 15%-30% interface (plasma membrane) was collected, diluted with TEDK as necessary and centrifuged at 105,000 x g for 1 hour. The pellet (plasma membrane) resulting from this centrifugation was suspended in TEDK, containing 1% protease inhibitor cocktail solution, as necessary.

Preparation of cytosol fraction The cells were collected as described above and centrifuged at 500 x g for 10 minutes. The cell pellet was suspended in 300 µl of TEDK, containing 1% protease inhibitor cocktail solution, and homogenized in a Dounce homogenizer (40 strokes). The cell suspension was centrifuged at 200 x g for 10 minutes. The supernatant

fluid was collected and centrifuged at 105,000 x g for 1 hour. The resulting supernatant (cytosol) was collected.

Preparation of nuclear fraction The cells were washed and collected in a chilled 1X PBS buffer solution. The cell suspension was centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 600 µl of Buffer A [10 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.3 M sucrose, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. Nonidet P-40 was added to 0.6% (vol/vol) and the tubes were vortexed at setting 10 with a Vortex mixer (Spectra-Physics, California) for 20 seconds. Nuclei were harvested by centrifugation of the samples at 7,200 x g for 30 seconds. The pellet (nuclei) was resuspended in 300 µl of Buffer B [20 mM Tris (pH 7.8), 5 mM $MgCl_2$, 320 mM KCl, 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. The samples were centrifuged at 13,500 x g for 15 minutes. The resulting supernatant (nuclear extract) was collected.

The purity of the cellular fractions was assessed by assaying for proteins known to be associated with cellular components. Lamin B (a nuclear-specific protein) immunoreactivity was determined by western blotting. The activity of adenylate cyclase (a plasma membrane-associated protein) was determined by the Biotrack™ cAMP competitive enzymeimmunoassay system (RPN 225, Amersham Pharmacia Biotech, New Jersey). For this experiment, ROS 17/2.8 cells were treated with either ethanol (as vehicle) or 50 µM forskolin (a known stimulator of adenylate cyclase) for 1 hour. Subcellular fractions were prepared and adenylate cyclase activity was determined. Adenylate cyclase activity was compared between forskolin-stimulated

and non-stimulated cells. Lactate dehydrogenase (LDH, a cytoplasmic-located protein) activity was determined according to a published procedure (Shonk and Boxer, 1964). The rate at which NADH is used up by LDH in the reaction (**pyruvate + NADH + H^+ ↔ lactate + NAD^+**) was determined by measuring the absorbance of the rate of the reaction mixture at 340 nm. To calculate the activity of LDH the following equation was used: **enzyme activity (IU/litre) = dA/dT x Factor**, where: dA/dT is the rate of change of absorbance. dA/dT is calculated by obtaining the slope from the linear portion of the absorbance vs time plot. The factor is equal to **$Vt.10^6/E.L.S.$**, where: Vt is the total reaction volume (ml); Vs is the sample volume (ml); E is the molar absorptivity (NADH: 6300 L/mol/cm); L is the path length (1 cm).

Immunoblotting For immunoblotting, cellular fractions were diluted 1:1 (vol/vol) in 2X sample buffer [12 mM Tris, 1.5 mM EDTA, 20 mM DTT, 2% SDS, 20% glycerol, and 0.2% bromophenol blue] and solubilized by boiling for 5 minutes. The samples were electrophoresed in a 12.5% SDS-polyacrylamide minigel for 90 minutes at 160 V in 1X running buffer (250 mM Tris, 10 M glycine, and 0.01% SDS). The proteins were transferred electrophoretically to a PVDF membrane at 0.60 A (constant voltage) for 2 hours at 4^0C in 1X transblot buffer (250 mM Tris, 10 M glycine). The blots were blocked with 2.5% non-fat dry milk in TBST (1 mM Tris, 15 mM NaCl and 0.05% Tween) overnight at 4^0C. Following blocking, the blots were probed with polyclonal antibodies for PKCα, βI, βII, γ, ζ or ε, for 1 hour at 37^0C. The blots were washed three times for 5 minutes at 37^0C in TBST and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase for 50 minutes at 37^0C. The blots were washed subsequently 2 times for 15 minutes at 37^0C in TBST.

Detection and data analysis PKC expression on western blots was detected using the LumiGlo chemiluminescent substrate kit (Kirkegaard & Perry Laboratories, Maryland) and blots were exposed to x-ray film (RX-U, FujiFilm) with Quanta III intensifying screens. Bands were quantified by using the Molecular Dynamics 300 A Computing Densitometer (Sunnyvale, California). The range of the linearity of PKC density measurements was calculated to be between 5 μg to 50 μg of total protein. To decrease variability in PKC density measurements between experiments the amount of total protein electrophoresed and the exposure time of blots to x-ray films were kept constant. PKCs levels were expressed relative to control. Statistical comparison were made by using *t*-test (Statistica, StatSoft) for independent sample means at 95% confidence level. The data is expressed as the mean ± the standard error of the mean.

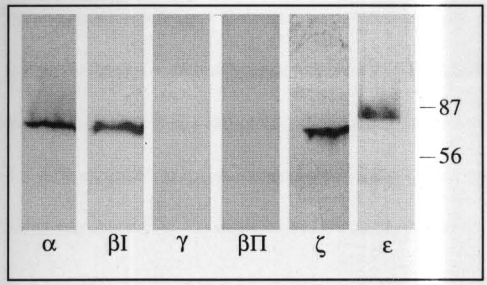
RESULTS

PKCα, βI, ζ and ε are expressed in ROS 17/2.8 cells

An important criteria to establish the relevance of PKC to the 1,25-$(OH)_2D_3$ intracellular signaling pathway is to show PKC expression and regulation in bone cells. First, ROS 17/2.8 cells were examined for PKC isoform expression. The results of these experiments are shown in figure 1. As shown in this figure, PKCα, βI, ζ and ε are present in ROS 17/2.8 cells, while PKCβII and PKCγ were not detected. It is apparent from the results of multiple experiments that PKCα and PKCζ are the most abundant isoforms in this cell line.

Figure 1. Expression of PKC isoforms in ROS 17/2.8 cells

ROS 17/2.8 cells were washed in chilled 1X PBS and collected in the same solution by using a cell scraper. The cell suspension was centrifuged at 500 x g for 10 minutes. The pellet for this centrifugation was electrophoresed on a 12.5% SDS-polyacrylamide minigel and western blot analysis was performed. Blots were probed with polyclonal antibodies for PKCα, βI, βII, ζ and ε using concentrations of 1:2000 for α, βI, ζ and ε and 1:250 for γ and βII. The membranes were incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase with concentrations of 1:8000 for the α, βI and ζ lines and 1:5000 for the ε, γ and βII lines. The PKCα and ζ lines were loaded with 1.0×10^6 cells each, PKCβI, βII, ε and γ lines were loaded with 2.0×10^6 cells each. PKC expression was detected using the ECL detection kit followed by autoradiography.


87
56
α
βI
γ
βΠ
ζ
ε

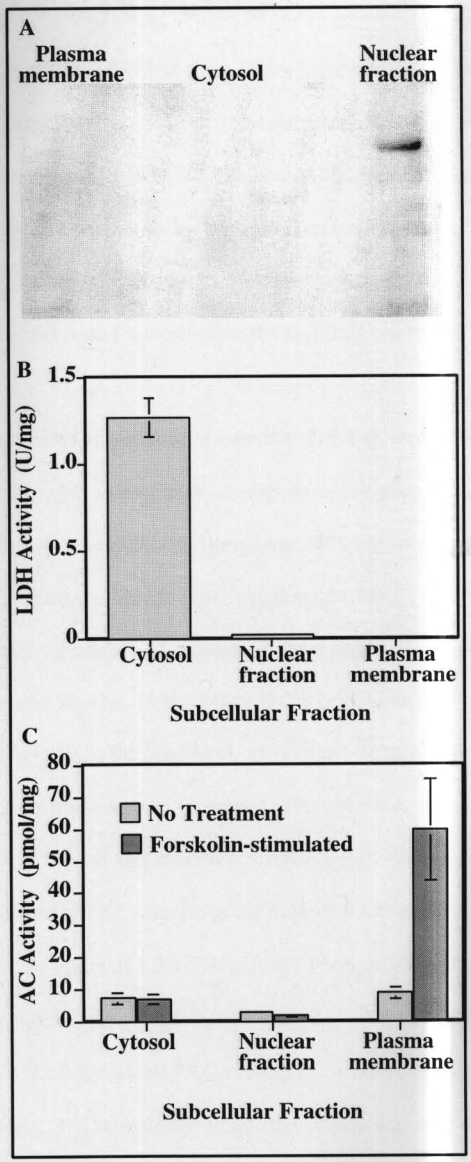
It is known that PKC isoenzymes are present predominantly in the cytoplasm in an inactive state and upon activation they translocate to other cellular compartments. 1,25-$(OH)_2D_3$ has been shown to induce translocation of PKC in several cell lines (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 2000). However, regulation of PKC activity by 1,25-$(OH)_2D_3$ in bone cells has not been reported. To assess whether 1,25-$(OH)_2D_3$ induces translocation of the expressed PKCs in ROS 17/2.8 cells, we determined PKCs subcellular localization by western blotting after treatment with 1,25-$(OH)_2D_3$. Cells were treated with ethanol (as control), 1,25-$(OH)_2D_3$ (0.05, 0.2, 2 and 20 nM), or with 100 nM PMA for the indicated amount of time. PMA is a known activator of the classical (α, βI, βII and γ) and novel [δ, ε, η(L), θ and μ] PKCs and it was used in the experiments as a positive control. Treatment with PMA was limited to 1 hour or less to avoid down regulation of PKC, as has been reported after prolonged treatment with phorbol esters (Szallsi et al., 1994, Keenan et al., 1995, Johnson et al., 1995, Sanders and Stern, 1996). After treatment, nuclear, plasma membrane and cytosolic fractions were prepared.

Purity of subcellular fractions

The potential of contamination of subcellular fractions, particularly the nuclei, has been a problem when analyzing translocation of PKC. To determine the purity of the cellular fractions Adenylate cyclase (a plasma membrane-associated protein) and lactate dehydrogenase (a cytoplasmic-located protein) activity, and lamin B (a nuclear-specific protein) immunoreactivity were determined. The results of these experiments are shown in figure 2. As shown in panel A, lamin B was detected only in the nuclear fraction. No lamin B immunoactivity was detected in the cytosol or plasma membrane. As shown in panel B, lactate dehydrogenase activity was

Figure 2. Characterization of cellular fraction

Cells were fractionated into nuclei, cytosol and plasma membrane as mentioned under materials and methods. Panel A: 20 μg of total protein from each fraction were electrophoresed on a 12.5% SDS- polyacrylamide minigel and Western blot analysis was performed. A 1:2000 dilution of a polyclonal antibody for lamin B (a nuclear-specific protein) and a 1:5000 dilution of anti-goat secondary antibody conjugated to HRP were used. Lamin B expression was detected using the ECL detection kit followed by autoradiography. This experiment was repeated twice. Panel B: Lactate dehydrogenase (LDH, a cytoplasmic-located protein) activity was determined according to published procedure (Shonk and Boxer, 1964). The error bars indicate the standard error of the mean of eight determinations. Panel C: Forskolin-stimulated adenylate cyclase (AC) activity was assayed by using the Biotrack™ cAMP competitive enzymeimmunoassay system (RPN 225) from Amershan Pharmacia Biotech (New Jersey). The error bars indicate the standard error of the mean of at least 6 determinations.


A
Plasma membrane
Cytosol
Nuclear fraction
B
LDH Activity (U/mg)
1.5
1.0
0.5
0
Cytosol
Nuclear fraction
Plasma membrane
Subcellular Fraction
C
AC Activity (pmol/mg)
80
70
60
50
40
30
20
10
0
No Treatment
Forskolin-stimulated
Cytosol
Nuclear fraction
Plasma membrane
Subcellular Fraction

mostly detected in the cytosol (1.27 U/μg). Very low lactate dehydrogenase activity (0.02 U/μg) was detected in the nuclear fraction. No lactate dehydrogenase activity was detected in the plasma membrane. Treatment of ROS 17/2.8 cells with 50 μM forskolin resulted in a 6.9-fold increase in adenylate cyclase activity in the plasma membrane compared to control cells (panel C). No significant change was observed for adenylate cyclase activity in the nuclear and cytosolic fractions obtained from forskolin-stimulated cells compared to non-stimulated cells.

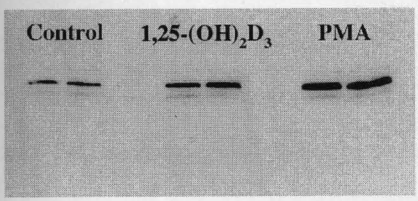
1,25-$(OH)_2D_3$ increased PKCα levels in the nuclear fraction in a time-dependent manner

PKCα was studied initially because of previous reports implicating 1,25-$(OH)_2D_3$ in its regulation (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 1999, Capiati et al., 2000). A 20 nM concentration of 1,25-$(OH)_2D_3$ was used because it resulted in near maximal stimulation of osteocalcin gene expression in previously performed experiments (Mosavin and Mellon, 1996). ROS 17/2.8 cells were treated with either ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$ for 5 minutes, 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours, and nuclear fractions were prepared. 100 nM PMA treatment was used as a positive control. PMA treatment was limited to 1 hour or less. Figure 3A represents an immunoblot for expression of PKCα levels in the nuclear fraction after 30 minutes treatment. Figure 3B summarizes the effect of 1,25-$(OH)_2D_3$ and PMA on PKCα levels at different times of treatment. As shown in this figure, treatment with 20 nM 1,25-$(OH)_2D_3$ for 15 minutes, 30 minutes, 1 hour and 24 hours increased PKCα levels in the nuclear fraction by 2.3-, 2.3-, 2.6- and 2.4-fold, respectively. No statistically significant change was observed with 1,25-$(OH)_2D_3$ treatment at 5 minutes, 6 hours and 12 hours compared to control. In comparison, treatment for

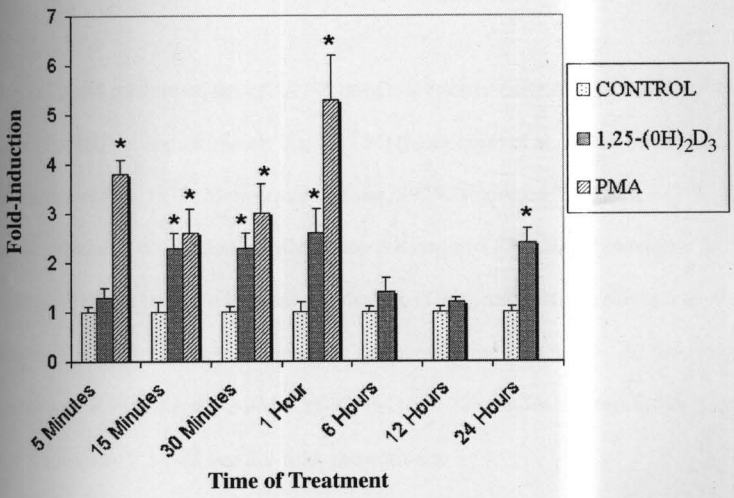
Figure 3. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα levels in the nuclear fraction

Panel A: Example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA. Nuclear fractions were prepared as described before. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed. A 1:2000 dilution of PKCα polyclonal antibody and a 1:8000 dilution of anti-rabbit secondary antibody conjugated to HRP were used. PKCα expression was detected using ECL detection kit followed by autoradiography. **Panel B**: Densitometric analysis of PKCα levels in the nuclei of ROS 17/2.8 cells after treatment with ethanol (as control) or 20 nM 1,25-$(OH)_2D_3$ for 5 minutes, 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours, or with 100 nM PMA for 5 minutes, 15 minutes, 30 minutes and 1 hour. PKCα levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control**. ND, not determined.

A.


Control
1,25-(OH)2D3
PMA

B.


Fold-Induction
0
1
2
3
4
5
6
7
5 Minutes
15 Minutes
30 Minutes
1 Hour
6 Hours
12 Hours
24 Hours
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

5 minutes, 15 minutes, 30 minutes and 1 hour with PMA significantly increased PKCα levels in the nuclear fraction by 3.8-fold, 2.6-fold, 3.0-fold and 5.3-fold, respectively.

The effect of 2 nM 1,25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction at 30 minutes, 1 hour and 24 hours was also determined. The results of these experiments are summarized in figure 4. As shown in this figure, treatment with 2 nM 1,25-$(OH)_2D_3$ increased PKCα levels by 1.8-fold, 1.9-fold and 2.7-fold, respectively.

2 nM and 20 nM concentrations of 1,25-$(OH)_2D_3$ are above the values of the K_D of the vitamin D receptor (7×10^{-11} to approximately 2×10^{-10} M) (Manolagas et al., 1980, Chen et al., 1979, Eisman and DeLuca, 1977, Mellon and DeLuca, 1979, Mellon and DeLuca, 1980). Therefore, we tested whether 0.05 nM and 0.2 nM concentrations of 1,25-$(OH)_2D_3$ could evoke changes in expression of PKCα levels in the nuclear fraction. Thirty minutes was selected as the time of treatment. The results of these experiments are summarized in figure 5. As shown in the figure, treatment with 0.05 nM and 0.2 nM 1,25-$(OH)_2D_3$ for 30 minutes increased PKCα levels in the nuclear fraction by 2.3-fold and 2.2-fold, respectively.

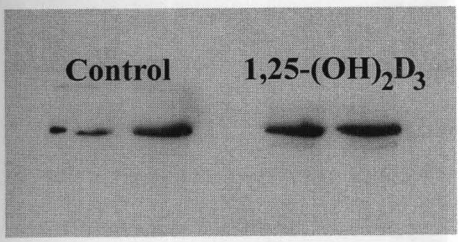
1,25-$(OH)_2D_3$ did not alter PKCζ, βI and ε levels in the nuclear fraction

Figure 6A shows an immunoblot for the expression of PKCζ in the nuclear fraction after ROS 17/2.8 cells were treated for 30 minutes with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA. Figure 6B summarizes the effect of 1,25-$(OH)_2D_3$ and PMA treatment on PKCζ levels in the nuclear fraction at 5 minutes, 15 minutes, 30 minutes and 1 hour. As shown in this figure, treatment with

Figure 4. Effect of 2 nM 1,25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction

Panel A: Example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control) or 2 nM 1,25-$(OH)_2D_3$. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCα levels in the nuclei of ROS 17/2.8 cells after treatment with ethanol (as control) or 2 nM 1,25-$(OH)_2D_3$ for 30 minutes, 1 hour and 24 hours. PKCα levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control.**

A.


Control
1,25-(OH)2D3

B.

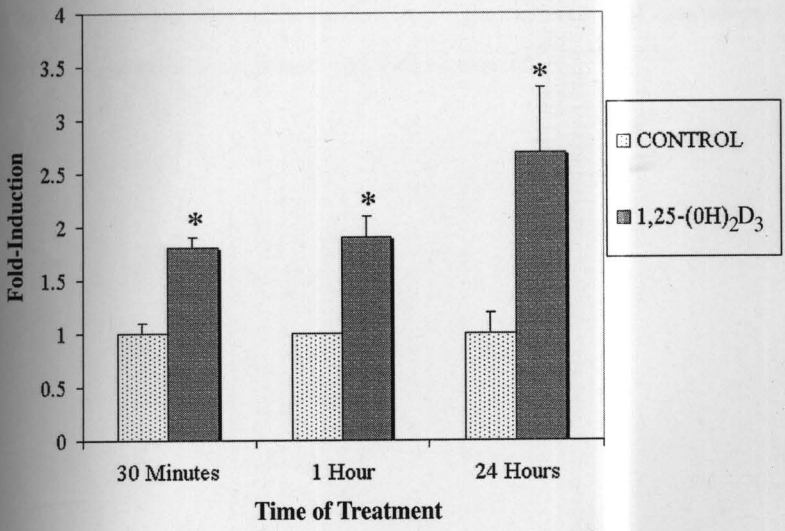
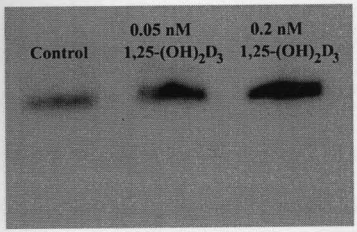

Fold-Induction
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
30 Minutes
1 Hour
24 Hours
Time of Treatment
CONTROL
1,25-(0H)2D3

Figure 5. Effect of 0.05 nM and 0.2 nM 1,25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction

Panel A: Example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control), or 0.05 nM 1,25-$(OH)_2D_3$, or 0.2 nM 1,25-$(OH)_2D_3$. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCα levels in the nuclei of ROS 17/2.8 cells after 30 minutes treatment. PKCα levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control**.

A.


Control
0.05 nM
1,25-(OH)2D3
0.2 nM
1,25-(OH)2D3

B.

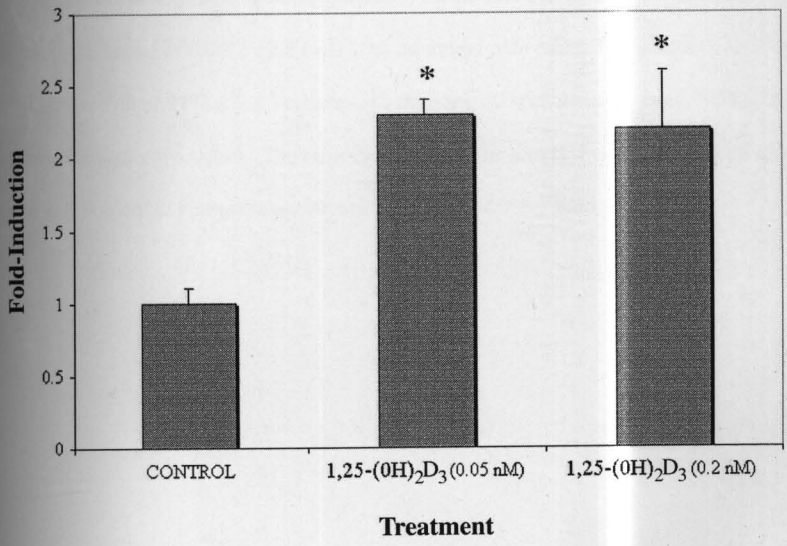
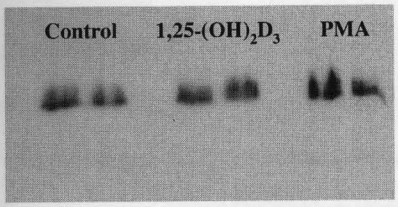

Fold-Induction
3
2.5
2
1.5
1
0.5
0
*
*
CONTROL
1,25-(0H)2D3 (0.05 nM)
1,25-(0H)2D3 (0.2 nM)
Treatment

Figure 6. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCζ levels in the nuclear fraction

Panel A: Example of immunoblot for the expression of PKCζ levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed. A 1:2000 dilution of PKCζ polyclonal antibody and a 1:8000 dilution of anti-rabbit secondary antibody conjugated to HRP were used. PKCζ expression was detected using ECL detection kit followed by autoradiography. **Panel B**: Densitometric analysis of PKCζ levels in the nuclei of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA for 5 minutes, 15 minutes, 30 minutes and 1 hour. PKCζ levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***$p < 0.05$ vs. control.**

A.


Control
1,25-(OH)2D3
PMA

B.

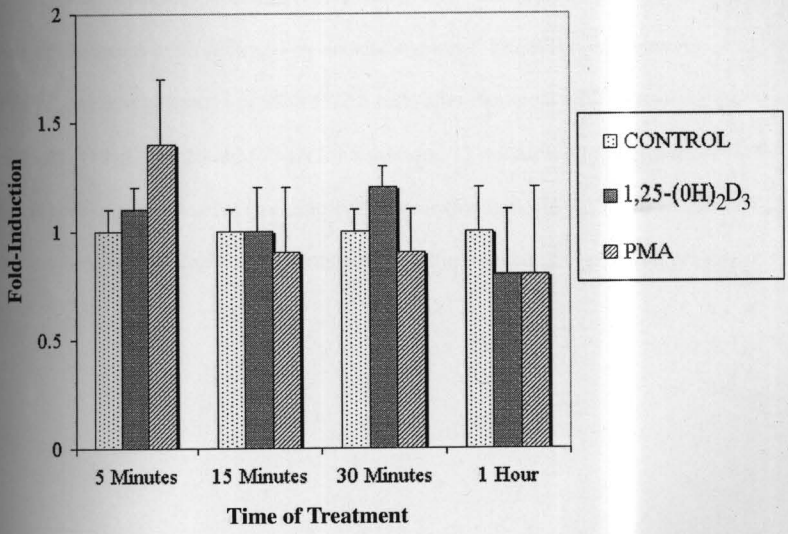
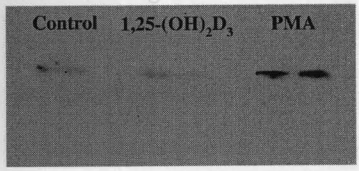

2
1.5
1
0.5
0
Fold-Induction
5 Minutes
15 Minutes
30 Minutes
1 Hour
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

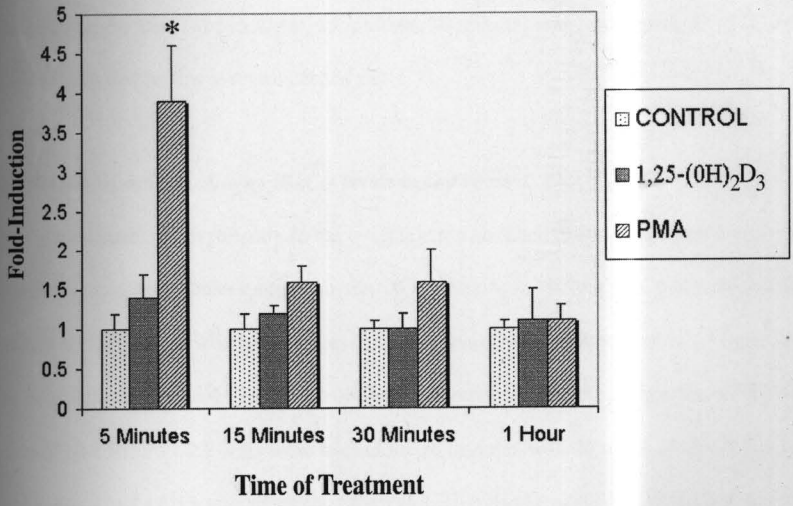
Figure 7. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCβI levels in the nuclear fraction

Panel A: Example of immunoblot for the expression of PKCβI levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed. A 1:2000 dilution of PKCβI polyclonal antibody and a 1:8000 dilution of anti-rabbit secondary antibody conjugated to HRP were used. PKCβI expression was detected using ECL detection kit followed by autoradiography. **Panel B**: Densitometric analysis of PKCβI levels in the nuclei of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA for 5 minutes, 15 minutes, 30 minutes and 1 hour. PKCβI levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control.**

A.


Control
1,25-(OH)2D3
PMA

B.


Fold-Induction
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
5 Minutes
15 Minutes
30 Minutes
1 Hour
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

either 1,25-$(OH)_2D_3$ or PMA did not significantly change PKCζ levels in the nuclear fraction at any time. The effect of 1,25-$(OH)_2D_3$ and PMA treatment on PKCβI levels in the nuclear fraction is summarized in figure 7. 1,25-$(OH)_2D_3$ did not alter PKCβI levels in the nuclear fraction at any time. PMA increased PKCβI nuclear levels by 3.9-fold at 5 minutes treatment. However, no significant change was detected at 15 minutes, 30 minutes or 1 hour. Negligible levels of PKCε were detected at all time points and were not altered by either 1,25-$(OH)_2D_3$ or PMA. Since we did not observe any statistically significant change on PKCζ and PKCβI levels in the nuclear fraction at 5 minutes, 15 minutes, 30 minutes and 1 hour with 20 nM 1,25-$(OH)_2D_3$, further testing was not carried out.

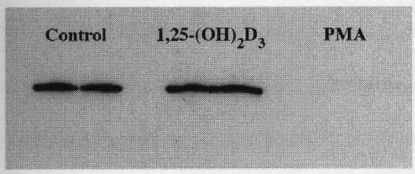
1,25-$(OH)_2D_3$ did not change PKCα levels in the cytosol

PKCs are present predominantly in the cytoplasm in an inactive state and upon activation they translocate to other cellular compartments. With the rise in nuclear PKCα accumulation, we sought to characterize whether changes in PKCα levels after treatment with 1,25-$(OH)_2D_3$ could be detected in the cytosol. Figure 8A shows an immunoblot for the expression of PKCα in the cytosol after ROS 17/2.8 cells were treated for 30 minutes with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA. Figure 8B summarizes the effect of 1,25-$(OH)_2D_3$ and PMA treatment on PKCα levels in the nuclear fraction at 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours. As shown in this figure, no statistically significant change was observed on PKCα levels at 30 minutes, 1 hour, 6 hours and 12 hours treatment with 1,25-$(OH)_2D_3$. However, a decrease of about 40% in PKCα levels was observed at 24 hours. In comparison, treatment for 30 minutes and 1 hour with PMA significantly decreased PKCα levels in the cytosol by over 90%.

Figure 8. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα levels in the cytosol

Panel A: Example of immunoblot for the expression of PKCα after 30 minutes treatment. Cytosolic fractions were prepared as described before. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCα levels in the cytosol of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$ for 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours, or with 100 nM PMA for 30 minutes and 1 hour. PKCα levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control.** ND, not determined. ∇ In the representative immunoblot depicted on panel A no PKCα band was observed after 30 minutes treatment with PMA. However, in other immunoblots a nearly negligible PKCα band was detected.

A.


Control
1,25-(OH)2D3
PMA

B.

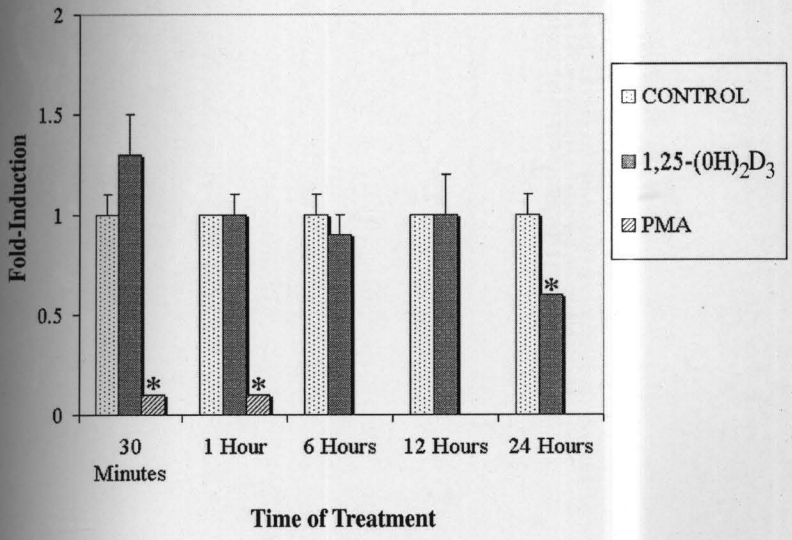
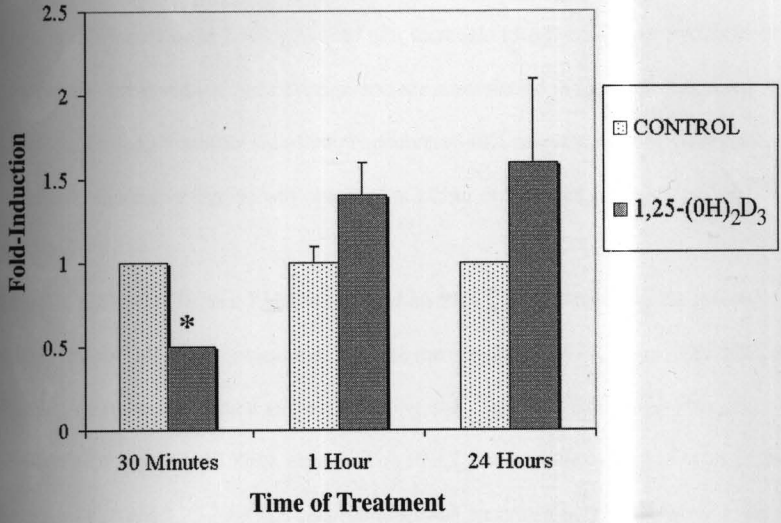

Fold-Induction
2
1.5
1
0.5
0
30 Minutes
1 Hour
6 Hours
12 Hours
24 Hours
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA
*
*
*

Figure 9. Effect of 2 nM 1,25-$(OH)_2D_3$ on PKCα levels in the cytosol

Densitometric analysis of PKCα levels in the cytosol of ROS 17/2.8 cells after treatment with ethanol (as control), or 2 nM 1,25-$(OH)_2D_3$ for 30 minutes, 1 hour and 24 hours. PKCα levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***$p < 0.05$ vs. control.**


2.5
2
1.5
1
0.5
0
Fold-Induction
*
30 Minutes
1 Hour
24 Hours
Time of Treatment
CONTROL
1,25-(0H)2D3

We also tested the effect of 2 nM 1,25-$(OH)_2D_3$ on PKCα levels in the cytosol at 30 minutes, 1 hour and 24 hours since 2 nM 1,25-$(OH)_2D_3$ treatment increased nuclear PKCα levels at these time points. The results of these experiments are summarized in figure 9. Treatment with 2 nM 1,25-$(OH)_2D_3$ for 30 minutes significantly decreased PKCα levels by 50%. However, no statistically significant change was observed at 1 hour or 24 hours.

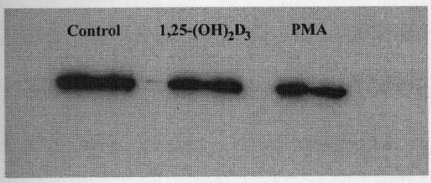
Effect of 1,25-$(OH)_2D_3$ and PMA treatment on PKCζ and βI levels in the cytosol

While 1,25-$(OH)_2D_3$ does not seem to regulate the expression of PKCζ and PKCβI in the nuclear fraction , we did measure their expression in cytosol. Figure 10 summarizes the effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA treatment on PKCζ levels in the cytosol. Figure 11 summarizes the effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA treatment on PKCβI levels in the cytosol. As shown in figure 10, treatment for 30 minutes by both 1,25-$(OH)_2D_3$ and PMA decreased PKCζ levels in the cytosol by 30% and 20%, respectively, while no significant change was observed at 1 hour. As shown in figure 11, treatment for 1 hour with 1,25-$(OH)_2D_3$ significantly decreased PKCβI levels by 40%, whereas no change was observed on PKCβI levels at 30 minutes. Treatment for 30 minutes and 1 hour with PMA decreased PKCβI levels by 90% and 80%, respectively.

Figure 10. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCζ levels in the cytosol

Panel A: Example of immunoblot for the expression of PKCζ after 30 minutes treatment. Cytosolic fractions were prepared as described before. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCζ levels in the cytosol of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA for 30 minutes and 1 hour. PKCζ levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control.**

A.


Control
1,25-(OH)2D3
PMA

B.

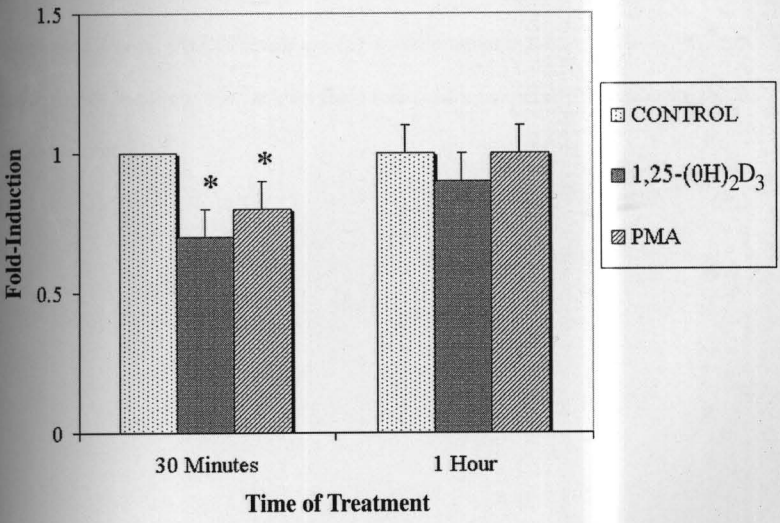
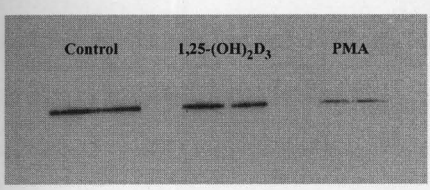

1.5
1
0.5
0
Fold-Induction
*
*
30 Minutes
1 Hour
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

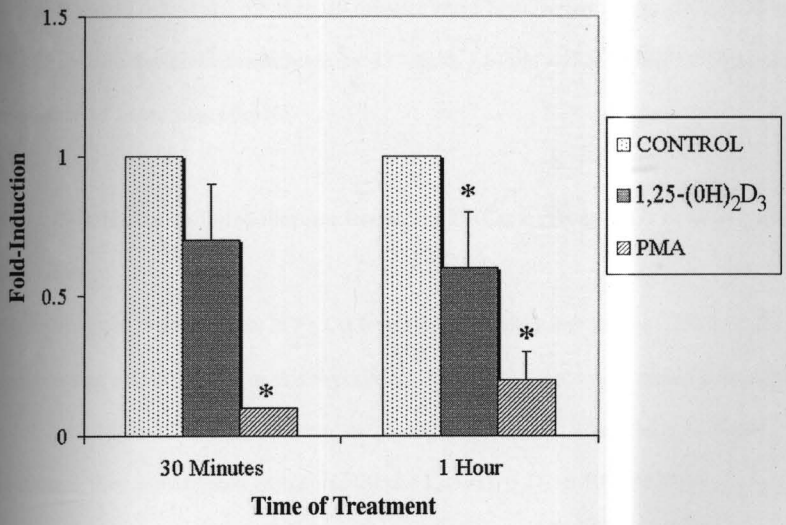
Figure 11. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCβI levels in the cytosol

Panel A: Example of immunoblot for the expression of PKCβI after 30 minutes treatment. Cytosolic fractions were prepared as described before. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCβI levels in the cytosol of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA for 30 minutes and 1 hour. PKCβI levels are expressed relative to control. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control.**

A.


Control
1,25-(OH)2D3
PMA

B.


1.5
1
0.5
0
Fold-Induction
*
*
30 Minutes
1 Hour
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

1,25-$(OH)_2D_3$ did not alter PKCα, PKCζ or PKCβI association with the plasma membrane

To determine translocation of PKCs to the plasma membrane, the effect of 5 minutes, 30 minutes and 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα, ζ and βI levels in the plasma membrane was examined. The results are summarized in figure 12. Treatment with 1,25-$(OH)_2D_3$ did not alter PKCα, ζ or βI levels in the plasma membrane at any time. As expected, treatment with PMA increased PKCα levels in the plasma membrane by 15.8-fold, 13.9-fold and 17.4-fold at 5 minutes, 30 minutes and 1 hour, respectively. PMA increased PKCβI levels in the plasma membrane by 22.2-fold, 1.9-fold and a 9.2-fold at 5 minutes, 30 minutes and 1 hour, respectively.

The 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels appears to be cell density-dependent

We investigated if the increase in PKCα levels in the nuclear fraction by 1,25-$(OH)_2D_3$ was dependent on cell-density. For this experiment, ROS 17/2.8 cells were plated at densities of 1.0 x 10^4, 1.8 x 10^4 and 3.6 x 10^4 cells/cm^2 on 55 cm^2 petri dishes. When the cells reached 50-70% confluence they were treated with either 20 nM 1,25-$(OH)_2D_3$ or 100 nM PMA. Twenty-four hours after treatment, nuclear fractions were prepared and western blot analysis was performed. This experiment was repeated twice. Figure 13 shows representative immunoblots for PKCα expression at 24 hours treatment. As shown in this figure, an increase in PKCα levels was observed when the cells were plated at 1.0 x 10^4 cells/cm^2, but not at higher densities (1.8 – 3.6 x 10^4 cells/cm^2). The PMA-mediated increase in nuclear PKCα levels was also abolished at higher cell densities.

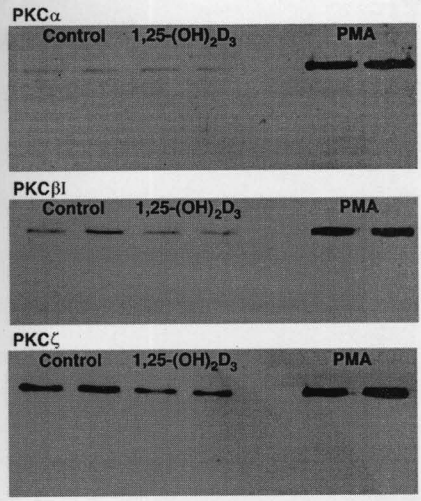
Effect of 25-OH-D_3 and 24(R),25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction

As shown previously, 1,25-$(OH)_2D_3$ selectively increased PKCα levels in the nuclear fraction. We determined the effectiveness of two other vitamin D_3 metabolites, 24(R),25-dihydroxyvitamin D_3 [24(R),25-$(OH)_2D_3$] and 25-hydroxyvitamin D_3 (25-OH-D_3), in increasing PKCα levels in the nuclei. 24(R),25-$(OH)_2D_3$ and 25-OH-D_3 are about 5000 and 1000 times less active than 1,25-$(OH)_2D_3$, respectively (Eisman and DeLuca, 1977, Mellon and DeLuca, 1980). We determined the effect of 30 minutes treatment with 100 nM and 250 nM 25-OH-D_3, and 500 nM and 1000 nM 24(R),25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction. This experiment was performed once. The results are summarized in figure 14. As shown in this figure, neither 24(R),25-$(OH)_2D_3$ nor 25-OH-D_3 altered PKCα nuclear levels.

Figure 12. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα, PKCβI and PKCζ levels in the plasma membrane

Panel A: Example of immunoblot for the expression of PKCα, PKCβI and PKCζ levels in ROS 17/2.8 cells after 5 minutes treatment. Plasma membrane fractions were prepared as described before. 30 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. **Panel B**: Densitometric analysis of PKCα, PKCβI and PKCζ levels in the plasma membrane of ROS 17/2.8 cells after treatment with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA for 5 minutes, 30 minutes and 1 hour. PKCs levels are expressed relative to control. The data represent at least two independent experiments performed in duplicates. The numbers in parenthesis represent the lower and higher values.

A.

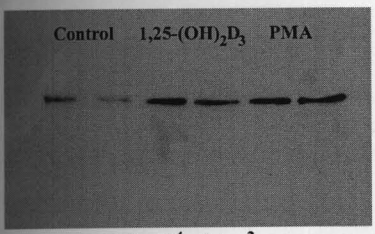


B.

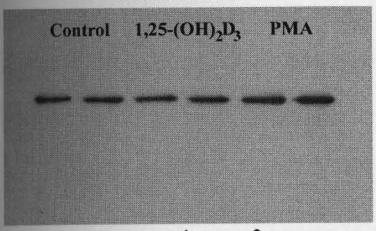
Plasma Membrane	Control	$1,25\text{-}(OH)_2D_3$	PMA
PKCα			
5 Minutes	1.0 (0.3, 1.6)	1.0 (0.1, 1.5)	15.8 (10.9, 22.6)
30 Minutes	1.0 (0.7, 1.3)	0.8 (0.3, 1.5)	13.9 (2.9, 29.5)
1 Hour	1.0 (0.6, 1.4)	0.9 (0.7, 1.1)	17.4 (14.2, 22.0)
PKCβI			
5 Minutes	1.0 (0.9, 1.1)	1.2 (0.5,1.9)	22.2 (4.9, 45.1)
30 Minutes	1.0 (0.9, 1.1)	1.0 (0.8, 1.2)	1.9 (1.0, 3.2)
1 Hour	1.0 (0.7, 1.3)	1.1 (0.7, 1.4)	9.2 (4.3, 12.7)
PKCζ			
5 Minutes	1.0 (0.8, 1.1)	0.9 (0.6, 1.3)	1.5 (1.3, 1.9)
30 Minutes	1.0 (0.8, 1.2)	0.8 (0.6, 1.0)	0.6 (0.4, 0.9)
1 Hour	1.0 (0.8, 1.1)	1.1 (0.1, 1.9)	1.0 (0.5, 1.6)

Figure 13. Effect of cell density on the 1,25-$(OH)_2D_3$-mediated increase in PKCα levels in the nuclear fraction

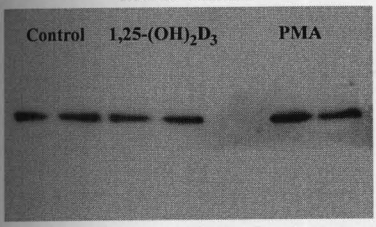
A representative example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells after 24 hours treatment. ROS 17/2.8 cells were plated at densities of 1.0 x 10^4 cells/cm^2, 1.8 x 10^4 cells/cm^2 and 3.6 x 10^4 cells/cm^2 on 55 cm^2 petri dishes. When the cells reached 50-70% confluence they were treated with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$, or 100 nM PMA. Twenty-four hours after treatment, nuclear fractions were prepared and western blot analysis was performed as described before.



1.0 x 10^4cells/cm^2



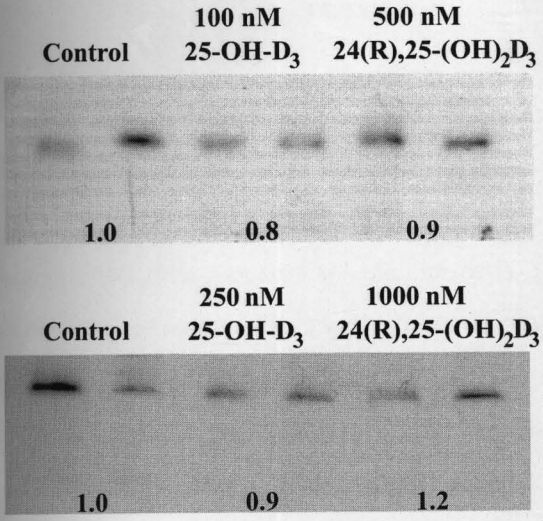
1.8 x 10^4cells/cm^2



3.6 x 10^4cells/cm^2

Figure 14. Effect of 25-OH-D_3 and 24(R),25-$(OH)_2D_3$ on PKCα levels in the nuclear fraction

A representative example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control), or 100 nM and 250 nM 25-OH-D_3, or 500 nM and 1000 nM 24(R),25-$(OH)_2D_3$. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed as described before. This experiment was performed once. The values at the bottom of the figure represent PKCα levels as expressed relative to control.


Control
100 nM 25-OH-D3
500 nM 24(R),25-(OH)2D3
1.0
0.8
0.9
Control
250 nM 25-OH-D3
1000 nM 24(R),25-(OH)2D3
1.0
0.9
1.2

DISCUSSION

We are interested in determining the signaling pathway(s) by which 1,25-$(OH)_2D_3$ regulates osteocalcin gene expression. Previous studies implicated PKC as a component of the signaling pathway of 1,25-$(OH)_2D_3$ in bone, particularly in the regulation of the osteocalcin gene (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996 #637). This led us to study PKC isoform expression and regulation by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. We determined that 1,25-$(OH)_2D_3$ specifically increased PKCα, but not PKCζ, βI or ε, levels in the nuclear fraction in a time-dependent manner. Whereas, 1,25-$(OH)_2D_3$ did not alter PKC levels of any of the isoforms in the plasma membrane. This 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels appears to be 1,25-$(OH)_2D_3$-specific since neither 24(R),25-$(OH)_2D_3$ nor 25-(OH)-D_3 altered PKCα levels in the nuclear fraction.

We first determined the expression of PKC isoforms in ROS 17/2.8 cells. ROS 17/2.8 cells have been used successfully as a model to study the effect of 1,25-$(OH)_2D_3$ on osteocalcin. This cell line exhibits several characteristics of a mature osteoblast: expression of vitamin D receptor, high responsiveness to parathyroid hormone, alkaline phosphate activity and the production of osteocalcin (Rodan and Majeska, 1983). We determined PKCα, βI, ε and ζ to be expressed in this cell line. PKCβII and PKCγ were not detected. These results are not surprising since both PKCα and PKCζ are known to be expressed universally, PKCβs are not expressed in all tissues and PKCγ has only been detected in the brain (Nishizuka, 1988). Our results coincide with previous data reported by Sanders and Stern (Sanders and Stern, 1996). They reported the expression of PKCα, βI, βII, ε and ζ in ROS 17/2.8 cells. We did not detect PKCβII. However,

the levels of PKCβII reported in their study were very low. In concordance with our results, they did not detect PKCγ in ROS 17/2.8 cells. PKCι also was found to be expressed, while PKCδ, η, and θ were not detected. We did not probe for those isoforms in our study. Of the PKC isoforms detected in our study, PKCζ and PKCα appear to be the most abundant ones.

We next investigated the effect of 1,25-$(OH)_2D_3$ on the translocation of the expressed PKC isoforms by immunoblotting. 1,25-$(OH)_2D_3$ has been reported to induce translocation of PKC isoforms in several cell lines (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 2000). To study PKC translocation by immunoblotting, we prepared subcellular fractions (cytosolic, plasma membrane and nuclei) and determined PKC isoform distribution after treatment with 1,25-$(OH)_2D_3$. The potential of contamination of subcellular fractions, particularly the nuclei, has been a problem when analyzing translocation of PKC. We determined the purity of the cellular fractions by assaying for proteins known to be associated with cellular components.

Treatment with 20 nM 1,25-$(OH)_2D_3$ increased PKCα levels, but not PKCβI, ε or ζ, in the nuclear fraction in a time-dependent manner. The 20 nM 1,25-$(OH)_2D_3$-mediated increase in PKCα levels in the nuclei appears to be a biphasic event. PKCα levels increased early (15 minutes), peaked at 1 hour, decreased to control levels at 6 and 12 hours, and then increased again at 24 hours. The significance of this biphasic response is not known. Nevertheless, the existence of a biphasic generation of diacylglycerol (DAG), a PKC activator, has been reported (Nishizuka, 1995). The first phase of DAG generation occurs early after treatment and disappears promptly. The generation of DAG in this phase is mediated by the hydrolysis of

phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). The second phase, that persists for minutes or hours, is mediated by the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) and/or phospholipase A_2 (PLA_2). 1,25-$(OH)_2D_3$ has been shown to increase mRNA and protein levels of some PLC isoforms (Pillai et al., 1995, Xie and Bikle, 1997), to regulate the activity of PLA_2 (Sylvia et al., 1998), and to regulate PLD activity and expression (Khare et al., 1999, Griner et al., 1999). In mouse epidermal keratinocytes treatment with 1,25-$(OH)_2D_3$ induced PLD-1 (a PLD isoenzyme) expression that was significant at 8 hours and peaked at 24 hours (Griner et al., 1999). Moreover, this effect was accompanied by an increase in total PLD-1 activity. Regulation of PLD-1 expression by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells could explain the induction of PKCα levels in the nuclei that was observed at 24 hours in our study.

1,25-$(OH)_2D_3$ has been shown to regulate PKC activity in several cell lines. In Madin Darby bovine kidney (MDBK) cells, treatment with 1,25-$(OH)_2D_3$ increased plasma membrane association of PKCα and induced translocation of PKCβ to the nuclei (Simboli-Campbell et al., 1994) . In human promyelocytic leukemia NB4 cells, 1,25-$(OH)_2D_3$ induced translocation of PKCδ to the nuclei, and PKCα to the nuclei and to a particulate fraction (Berry et al., 1996). However, in these studies relatively high concentrations of 1,25-$(OH)_2D_3$ were used (100 and 200 nM, respectively). In our studies doses of 2 nM and 20 nM 1,25-$(OH)_2D_3$ increased PKCα levels in the nuclear fraction. Moreover, an increase in PKCα levels in the nuclear fraction was also observed with 1,25-$(OH)_2D_3$ doses as low as 0.2 and 0.05 nM. These 1,25-$(OH)_2D_3$ doses are between the values of the K_D of the vitamin D receptor (7×10^{-11} to approximately 2×10^{-10} M) (Manolagas et al., 1980, Chen et al., 1979, Eisman and DeLuca, 1977, Mellon and DeLuca,

1979, Mellon and DeLuca, 1980), indicating the physiological relevance of the 1,25-$(OH)_2D_3$-mediated PKCα increase in ROS 17/2.8 cells.

The effect of vitamin D_3 analogues on PKC activity has been reported. Sylvia et al. examined the effect of the 24(R),25-$(OH)_2D_3$ on PKC activity in isolated plasma membranes and extracellular matrix vesicles of resting zone chondrocytes and growth zone chondrocytes (Sylvia et al., 1996). PKCα is the major isoform in cell layer lysates as well as in plasma membrane isolated from both cell types while PKCζ is the predominant form in matrix vesicles. 10^{-8} M - 10^{-7} M 24(R),25-$(OH)_2D_3$ inhibited matrix vesicle PKC, but stimulated plasma membrane PKC when added directly to the isolated membrane fraction. The same results were obtained with 1,25-$(OH)_2D_3$ (10^{-9} M – 10^{-8} M). In another study by Bissonnette et al. 10 nM 1,25-$(OH)_2D_3$ caused translocation of PKCα from the soluble to the particulate fraction in human colon cancer-derived Caco-2 cells (Bissonnette et al., 1994). However, 25-OH-D_3 and 24(R),25$(OH)_2D_3$, over a range of concentrations as high as 1 μM, did not affect the distribution of PKCα. In our studies 25-OH-D_3 and 24(R),25-$(OH)_2D_3$ in concentrations as high as 500 nM and 1000 nM, respectively, failed to increase PKCα levels in the nuclear fraction, suggesting that the PKCα increase in nuclear levels in ROS cells 17/2.8 is 1,25-$(OH)_2D_3$ specific.

We were not able to certainly detect a decrease in cytosolic PKCα levels by 1,25-$(OH)_2D_3$ treatment. The increase in nuclear PKCα levels may indicate that 1,25-$(OH)_2D_3$ induced the synthesis of PKCα since 1,25-$(OH)_2D_3$ is known to regulate gene expression. The regulation of PKCα and PKCβ gene expression by 1,25-$(OH)_2D_3$ has been reported by Obeid and Solomon (Obeid et al., 1990, Solomon et al., 1991). Both studies reported an increase of over 4-fold in

PKCα and PKCβs mRNA levels after 24 hours of treatment with 1,25-$(OH)_2D_3$. However, while Obeid and coworkers reported increases in transcriptional rates for these isoforms after treatment with 1,25-$(OH)_2D_3$, Solomon and coworkers were not able to detect changes on the PKCα, βI and βII rates of transcription. The apparent discrepancy may be due to the fact that to normalize the rate of transcription of PKCβs and PKCα genes different internal control genes were used between the studies. It is also worth mentioning that relatively high concentrations 1,25-$(OH)_2D_3$ were used (100 nM and 600 nM, respectively) in these studies. These concentrations are above the physiological concentrations of 1,25-$(OH)_2D_3$, and may be of no physiological relevance in respect to the regulation of PKC gene expression. In the appendix section we will discuss research directed to study the regulation of PKCα gene expression by 1,25-$(OH)_2D_3$ by RT-PCR. The data suggests that 1,25-$(OH)_2D_3$ does not significantly regulate PKCα gene expression in ROS 17/2.8 cells. On the other hand, we were able to detect a decrease in cytosolic PKCα levels by immunofluorescence. That data is discussed in chapter 3.

1,25-$(OH)_2D_3$ treatment did not alter PKCζ and PKCβI levels in the nuclear and plasma membrane fractions. However, decreases in cytosolic PKCζ and PKCβI levels were observed at 30 minutes and 1 hour of treatment, respectively, with 1,25-$(OH)_2D_3$. This may indicate that 1,25-$(OH)_2D_3$ induced translocation of these two isoforms to other cellular compartments not examined in this study since no increase in PKCζ or PKCβI levels was observed in the nuclear fraction or plasma membrane.

PMA, a known activator of conventional and novel PKCs (Nishizuka, 1995), was used in our experiments as a positive control. As expected, treatment with PMA caused translocation of

PKCα to the plasma membrane and nuclear fraction at all the time points examined. This was accompanied by a concomitant decrease in PKCα levels in the cytosol. PMA treatment decreased PKCβI levels in the cytosol at 30 minutes and 1 hour with a concomitant translocation to the plasma membrane. On the other hand, PMA treatment for 30 minutes decreased PKCζ levels in the cytosol and plasma membrane. This regulation of PKCζ by PMA may be cell specific or secondary to the phorbol ester effect on the conventional and novel PKC since PKCζ has been suggested not to be regulated by phorbol esters (Nakanishi and Exton, 1992, Ono et al., 1989, Liyanage et al., 1992, Ways et al., 1992). Nevertheless, down-regulation of this isoform by phorbol ester has been previously reported in adipocytes (Standaert et al., 1993), cardiac myocytes (Johnson et al., 1995), human platelets (Crabos et al., 1991), and in kidney cells (Simboli-Campbell et al., 1994).

Previous studies have implicated PKC signaling pathway in the 1,25-$(OH)_2D_3$-mediated regulation of the osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). In this chapter we report that 1,25-$(OH)_2D_3$ specifically increased PKCα in the nuclear fraction, suggesting a role for this isoform in 1,25-$(OH)_2D_3$-mediated nuclear events. To our knowledge this is the first study that reports regulation of PKC by 1,25-$(OH)_2D_3$ in bone cells.

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Chapter III

Immunofluorescence analyis of the effect of 1,25-dihydroxyvitamin D_3 on protein kinase Cα subcellular localization in rat osteoblast cells (ROS 17/2.8)

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ABSTRACT

We determined PKCα subcellular distribution in ROS 17/2.8 cells after 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA treatment by epi-fluorescence and confocal microscopy. In this chapter we report that 1,25-$(OH)_2D_3$ increased PKCα fluorescence in the nucleus in a time-dependent manner. In accord with the immunoblotting data, the percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence, as detected by epi-fluorescence, was higher for 1,25-$(OH)_2D_3$-treated cells at 30 minutes,1 hour and 24 hours, but not at 6 and 12 hours. The 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα fluorescence was accompanied by a decrease in PKCα cytosolic levels, indicating PKCα translocation. Confocal microscopy analysis corroborated that the PKCα signal detected in the nuclei by epi-fluorescence truly reflected PKCα nuclear localization. On the other hand, immunofluorescence microscopy revealed differences in the regulation of PKCα by 1,25-$(OH)_2D_3$ and PMA. While 1,25-$(OH)_2D_3$ increased intranuclear PKCα, PMA increased PKCα that appears to be associated with the perinucleus and/or with structures encompassing the nuclei. To our knowledge, this is the first study that reports 1,25-$(OH)_2D_3$-mediated translocation of a PKC isoform in a bone cell line.

INTRODUCTION

PKC is a family of serine/threonine kinases that consists of at least 12 isoforms. These isoforms are involved in the transduction response to a vast array of agonists such as neurotransmitters, growth factors and hormones (Dekker and Parker, 1994, Nishizuka, 1995). PKCs are mostly present in the cytoplasm in an inactive state. They are activated by DAG which is generated through agonist-stimulated hydrolysis of membrane phospholipids. Once activated, PKCs translocate to the plasma membrane where they regulate the catalytic activity of target proteins through phosphorylation on serine and threonine residues. Translocation to other cellular compartments, including the nuclei, has been reported (Chida et al., 1994, Disatnik et al., 1994, Buchner, 1995, Goodnight et al., 1995, Kiley and Parker, 1995, Prestle et al., 1996, Westermann et al., 1996).

In the previous chapter we employed biochemical studies to determine PKC expression and regulation by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. We reported that 1,25-$(OH)_2D_3$ specifically increased PKCα in the nuclear fraction in a time-dependent manner. However, by subcellular fractionation contamination of the isolated nuclei with components from other cellular fractions is possible. Even though we determined that these subcellular fractions were relatively pure, cross-contamination cannot be ruled out. To further substantiate the biochemical data, we determined by epi-fluorescence and confocal microscopy the effect of 20 nM 1,25-$(OH)_2D_3$ treatment on PKCα subcellular localization.

In this chapter we report that 20 nM 1,25-$(OH)_2D_3$ increased PKCα nuclear fluorescence in ROS 17/2.8 cells in a time-dependent manner, as detected by epi-fluorescence. Moreover, the 1,25-$(OH)_2D_3$ mediated increase in nuclear PKCα fluorescence was accompanied by a concomitant decrease in the PKCα signal in the cytosol, indicating translocation.

Epi-fluorescence has many advantages over biochemical procedures including sensitivity. However, in a conventional epi-fluorescence microscope, the entire fluorescent specimen is exposed to excitation light and the emission collected by the objectives comes from throughout the specimen, including above and below the focal plane (Herman, 1998). This superimposition of detail within the plane of focus can create out-of-focus information that obscures structural detail. For instance, a fluorescence signal detected in a cellular compartment (e.i. nuclei) may not necessary indicate real fluorescence. Confocal microscopy was employed in our study to overcome this problem.

In confocal microscopy out-of-focus information is removed by illuminating a single point of the specimen with a focus beam (so that illumination intensity drops off rapidly above and below the plane of focus), and by placing a pin-hole aperture in the emission light pathway at a focal plane conjugate to the specimen (Herman, 1998). For the confocal analysis, consecutive optical sections (Z-series) separated by approximately 0.6 μm were taken. We determined that the PKCα signal detected by epi-fluorescence after 1,25-$(OH)_2D_3$ treatment truly reflected PKCα nuclear fluorescence.

Previous studies have implicated PKC signaling pathway in 1,25-$(OH)_2D_3$-mediated regulation of the osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). In this chapter we report that 1,25-$(OH)_2D_3$ translocates PKCα to the nuclear fraction, supporting a role for this isoform in the regulation of osteocalcin gene expression.

MATERIALS AND METHODS

Materials 1,25-$(OH)_2D_3$ was kindly provided by Dr. Hector F. DeLuca (University of Wisconsin-Madison). Rabbit polyclonal IgG antibody that recognizes PKCα was obtained from Santa Cruz Biotechnology (Santa Cruz, California). cPKCα antibody was raised against a peptide mapping at the carboxy terminus of cPKCα of human origin. cPKCα antibody recognizes PKCα of mouse, rat and human origin. Fluorescein-conjugated anti-rabbit IgG [F(ab')2] antibody was obtained from Pierce (Rockford, Illinois). This antibody reacts with the heavy chains on rabbit IgG and with the light chains common to most rabbit immunoglobulins.

Cell culture ROS 17/2.8 cells were maintained in Ham's F-12 (1:5) culture medium supplemented with 5% (vol/vol) defined fetal bovine serum (Hyclone, Utah), penicillin (100 u/ml), and streptomycin (0.1 mg/ml). Cells were propagated continuously at 37^0C under a humidified 5% CO_2: 95% air mixture. For the experiments, cells were plated at a density of 1.0 x 10^4cells/cm^2 on 15 mm glass coverslips and allowed to adhere overnight. When the cells reached 50-70% confluence they were treated with either ethanol (as control), 1,25-$(OH)_2D_3$ (20 nM) or PMA (100 nM) for a specified amount of time. Experiments were carried out on cells between the 5th and 30th passage.

Immunofluorescence Immunofluorescence microscopy was performed by modification of a published procedure (Buchner et al., 1997). Briefly, cells were allowed to adhere to coverslips and were treated with ethanol (as vehicle), or 1,25-$(OH)_2D_3$ (20 nM), or PMA (100 nM) for a specified amount of time. Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.05% saponin in 1X PBS for 5 minutes and blocked with 1% BSA in 1X PBS for 30 minutes. Cells were incubated with either a rabbit polyclonal antibody that recognizes PKCα (diluted 1:10 in 0.05% saponin, 0.1% BSA, 300 mM glucose in 1X PBS solution, for a concentration of 20 μg/ml), or with rabbit-IgG (20 μg/ml), overnight at 4^0C. Rabbit-IgG was used to determine nonspecific fluorescence (negative control). Cells were then incubated for two hours with FITC-conjugated anti-rabbit secondary antibody (diluted 1:50 in 0.05% saponin, 0.1% BSA, 300 mM glucose in 1X PBS solution, for a concentration of 20 μg/ml). Coverslips were mounted on slides and fluorescence was observed using either, a Nikon diaphot inverted microscope with a Nikon Dual Optical Path Tube connected to Photometrics PXL cooled CCD and Newvicon cameras, or a BioRad MRC-600 confocal microscope equipped with a mixed gas (Ar/Ak) laser with 488 nm, 568 nm and 647 nm excitation wavelengths. For confocal microscopy analysis, consecutive optical sections (Z-series) separated by approximately 0.6 μm were taken.

Data analysis. The percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence after treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA at various times was determined. For this, at least 250 cells were counted per treatment in each of the time points examined. Statistical comparison were made by using *t*-test (Statistica, StatSoft) for independent sample means at 95%

confidence level. The data is expressed as the mean ± the standard error of the mean, unless otherwise stated.

RESULTS

Epi-fluorescence Analysis

In the previous chapter we presented immunoblotting data demonstrating that 1,25-$(OH)_2D_3$ specifically increases PKCα levels in the nuclei. To further substantiate the effect of 1,25-$(OH)_2D_3$ on PKCα levels, immunofluorescence microscopy was used a second line of investigation. For these experiments, ROS 17/2.8 cells were plated on coverslips and allowed to reach 50-70% confluence. At this point the cells were treated with ethanol (as control), or 20 nM 1,25-$(OH)_2D_3$ for 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours, or 100 nM PMA for 30 minutes and 1 hour. Immunofluorescence microscopy was performed as described under Materials and Methods.

Treatment with 20 nM 1,25-$(OH)_2D_3$ for 30 minutes translocated PKCα to the nuclei

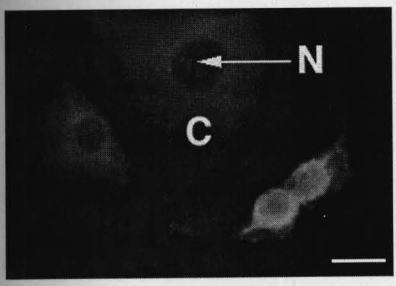
Figure 1 shows fluorescent images of PKCα in ROS 17/2.8 cells after 30 minutes treatment with ethanol (as control) and 20 nM 1,25-$(OH)_2D_3$. As shown in this figure, 1,25-$(OH)_2D_3$ increased PKCα fluorescence in the nuclei at 30 minutes. The increase in PKCα nuclear fluorescence was accompanied by a decrease in the cytosolic PKCα signal. On the other hand, most PKCα fluorescence was concentrated in the cytosol in control-treated cells. IgG fluorescence (negative control) was negligible.

Figure 1. Immunofluorescence distribution of PKCα in ROS 17/2.8 cells after 30 minutes treatment with 20 nM 1,25-$(OH)_2D_3$

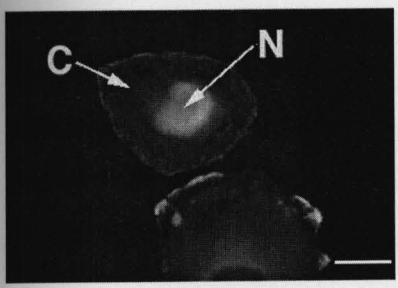
ROS 17/2.8 cells were allowed to adhere on coverslips overnight and were treated with ethanol (as control) or 20 nM 1,25-$(OH)_2D_3$ for 30 minutes. Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.05% saponin in 1X PBS for 5 minutes and blocked with 1% BSA in 1X PBS for 30 minutes. Cells were incubated with a polyclonal antibody for PKCα (20 µg/ml) overnight. Cells were incubated with anti-rabbit secondary antibody conjugated to FITC (20 µg/ml) for 2 hours. Immunofluorescence was observed using a Nikon diaphot inverted microscope. Strong nuclear PKCα fluorescence is observed in a 1,25-$(OH)_2D_3$-treated cell. In vehicle-treated cells most PKCα fluorescence is concentrated in the cytosol. Negative control: rabbit-IgG. Nuclei (N), cytosol (C). *Scale Bar* = 15 µm



Negative Control



Vehicle



1,25-$(OH)_2D_3$

Treatment with 20 nM 1,25-$(OH)_2D_3$ for 1 hour translocated PKCα to the nuclei

Figure 2 shows PKCα fluorescence in ROS 17/2.8 cells after 1 hour treatment with ethanol (as control) and 20 nM 1,25-$(OH)_2D_3$. As with 30 minutes treatment, 1 hour treatment with 1,25-$(OH)_2D_3$ increased PKCα fluorescence in the nuclei. This increase in nuclear PKCα levels was also accompanied by a decrease in cytosolic PKCα levels. In control-treated cells PKCα fluorescence was mostly localized in the cytosol. IgG fluorescence (negative control) was negligible.

Effect of 20 nM 1,25-$(OH)_2D_3$ on PKCα fluorescence at 6 and 12 hours treatment

Figure 3 shows fluorescent images of PKCα in ROS 17/2.8 cells after 6 and 12 hours treatment with ethanol (as control) and 20 nM 1,25-$(OH)_2D_3$. A shown in the figure, in 1,25-$(OH)_2D_3$-treated cells no PKCα fluorescence was detected in the nuclei at 6 or 12 hours. As in control-treated cells, PKCα fluorescence in 1,25-$(OH)_2D_3$-treated cells was mostly detected in the cytosol at both time points.

Effect of 20 nM 1,25-$(OH)_2D_3$ on PKCα fluorescence at 24 hours treatment

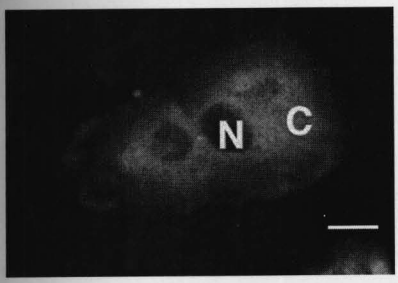
Figure 4 shows fluorescent images of PKCα in ROS 17/2.8 cells after 24 hours treatment with ethanol (as control) and 20 nM 1,25-$(OH)_2D_3$. As shown in this figure, PKCα fluorescence was mostly detected in the cytosol in both control- and 1,25-$(OH)_2D_3$-treated cells. PKCα fluorescence was also detected in the nuclei in 1,25-$(OH)_2D_3$-treated cells. However, the percentage of 1,25-$(OH)_2D_3$-treated cells showing PKCα nuclear fluorescence at 24 hours was not as high as observed at 30 minutes and 1 hour treatment.

Figure 2. Immunofluorescence distribution of PKCα in ROS 17/2.8 cells after 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$

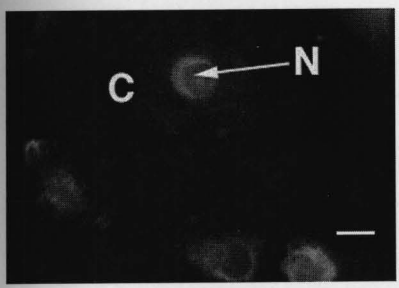
ROS 17/2.8 cells were allowed to adhere on coverslips overnight and were treated with ethanol (as control) or 20 nM 1,25-$(OH)_2D_3$ for 1 hour. Immunofluorescence was observed using a Nikon diaphot inverted microscope. Strong nuclear PKCα fluorescence is observed in 1,25-$(OH)_2D_3$-treated cells. Note the decrease in cytosolic PKCα levels in 1,25-$(OH)_2D_3$-treated cells compared to control. Negative control: rabbit-IgG. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm



Negative Control



Vehicle



1,25-$(OH)_2D_3$

Figure 3. Immunofluorescence distribution of PKCα in ROS 17/2.8 cells after 6 and 12 hours treatment with 20 nM 1,25-$(OH)_2D_3$

ROS 17/2.8 cells were allowed to adhere on coverslips overnight and were treated with ethanol (as control) or 20 nM 1,25-$(OH)_2D_3$ for 6 hours (**Panel A**) or 12 hours (**Panel B**). Immunofluorescence was observed using a Nikon diaphot inverted microscope. Note that PKCα fluorescence is mostly concentrated in the cytosol in 1,25-$(OH)_2D_3$-treated cells at both time points. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm

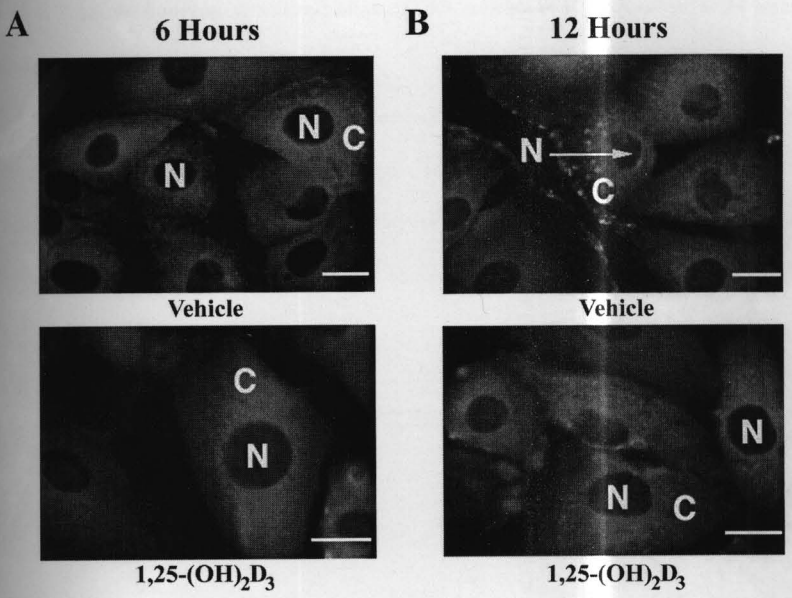
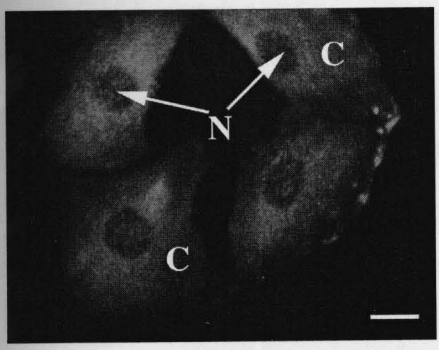
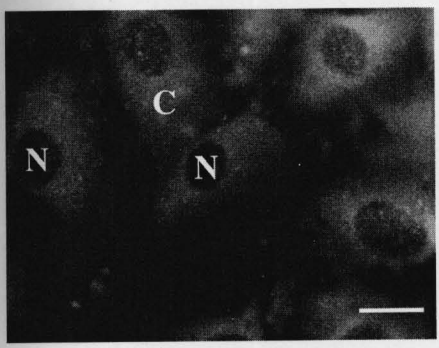

A
6 Hours
B
12 Hours
N
C
N
N
C
Vehicle
Vehicle
C
N
N
N
C
1,25-(OH)2D3
1,25-(OH)2D3

Figure 4. Immunofluorescence distribution of PKCα in ROS 17/2.8 cells after 24 hours treatment with 20 nM 1,25-$(OH)_2D_3$

ROS 17/2.8 cells were allowed to adhere on coverslips overnight and were treated with ethanol (as control) or 20 nM 1,25-$(OH)_2D_3$ for 24 hours. Immunofluorescence was observed using a Nikon diaphot inverted microscope. PKCα fluorescence is mostly concentrated in the cytosol in both control- and 1,25-$(OH)_2D_3$-treated cells. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm



Vehicle



1,25-$(OH)_2D_3$

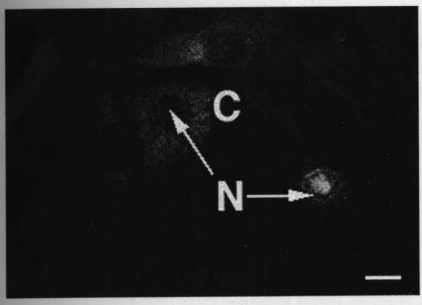
Treatment with 100 nM PMA for 30 minutes and 1 hour translocated PKCα to the nuclear membrane

Figure 5 shows fluorescent images of PKCα in ROS 17/2.8 cells after 30 minutes and 1 hour treatment with 100 nM PMA. As shown in the figure, PKCα fluorescence is localized around the nuclei. This pattern was observed in the majority of PMA-treated cells at both time points. On the contrary, few cells showed intranuclear PKCα fluorescence.

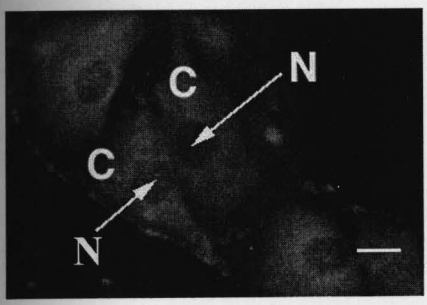
The percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence after treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA at different times was determined. For this at least 250 cells were counted per treatment at each of the time points examined. The results are summarized in figure 6. As shown in this figure, 40% and 34% of ROS 17/2.8 cells treated with 1,25-$(OH)_2D_3$ showed nuclear PKCα fluorescence at 30 minutes and 1 hour, respectively. On the other hand, only 10% and 18% of ethanol-treated cells showed nuclear PKCα fluorescence, respectively. However, no significant difference in the percentage of cells showing nuclear PKCα fluorescence was observed between 1,25-$(OH)_2D_3$-treated cells and ethanol-treated cells at 6 and 12 hours. At 24 hours, 13% of the 1,25-$(OH)_2D_3$-treated cells showed nuclear PKCα fluorescence, while 6% of ethanol-treated cells showed nuclear fluorescence. In comparison, 13% and 25% of the cells treated with PMA showed intranuclear PKCα fluorescence at 30 minutes and 1 hour, respectively. However, the majority of PMA-treated cells showed PKCα fluorescence around the nuclei instead of intranuclear.

Figure 5. Immunofluorescence distribution of PKCα in ROS 17/2.8 cells after 30 minutes and 1 hour treatment with 100 nM PMA

ROS 17/2.8 cells were allowed to adhere on coverslips overnight and were treated with 100 nM PMA for 30 minutes and 1 hour. Immunofluorescence was observed using a Nikon diaphot inverted microscope. Note that PKCα fluorescence is localized around the nuclei instead of intranuclear. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm



30 Minutes



1 Hour

Figure 6. Percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence

The percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence after treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA was determined.

For 30 minutes, 1 hour and 24 hours treatment the data is expressed as the mean of the percentage ± standard error of the mean of the percentage of ROS 17/2.8 cells showing nuclear PKCα fluorescence as determined by epi-fluorescence in three independent experiments. At least 250 cells were counted per treatment in each of the time points examined. ***P<.05 vs. control**

For 6 and 12 hours treatment the data is expressed as the mean of the percentage of ROS 17/2.8 cells showing nuclear PKCα as determined by epi-fluorescence in two independent experiments. At least 250 cells were counted per treatment in each of the time points examined. The numbers in parenthesis represents the lower and higher values of two determinations.

	ETHANOL	1,25-$(OH)_2D_3$	PMA
30 Minutes	10.0% ± 2.2%	**40.4% ± 7.9%***	12.8% ± 3.4%
1 Hour	13.1% ± 4.3%	**35.6% ± 5.8%***	13.3% ± 6.3%
6 Hours	1.5% (0%, 3.0%)	1.5% (0.8%, 2.3%)	ND
12 Hours	0.3% (0%, 0.7%)	1.7% (2.1%, 1.4%)	ND
24 Hours	6.4% ± 3.9%	13.0% ± 6.0%	ND

Confocal Microscopy Analysis

To confirm that the PKCα signal detected in the nuclei by epi-fluorescence truly reflected nuclear PKCα, we studied PKCα fluorescence by confocal microscopy at 30 minutes, 1 hour and 24 hours treatments.

PKCα fluorescence is detected in the nuclei in 1,25-$(OH)_2D_3$-treated cells at 30 minutes and 1 hour

Figure 7 shows optical sections of immunostained ROS 17/2.8 cells showing PKCα fluorescence distribution after 30 minutes treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. As shown in this figure, PKCα fluorescence is certainly detected in the nuclei with 1,25-$(OH)_2D_3$ treatment. On the contrary, PKCα fluorescence in the nuclei in the majority of ethanol-treated cells was negligible. The same results were obtained at 1 hour treatment with 1,25-$(OH)_2D_3$. As shown in Figure 8, treatment with 1,25-$(OH)_2D_3$ for 1 hour increased PKCα fluorescence in the nuclei compared to control. In control-treated cells the PKCα signal was concentrated in the cytosol. IgG fluorescence (negative control) was nearly negligible at both 30 minutes and 1 hour.

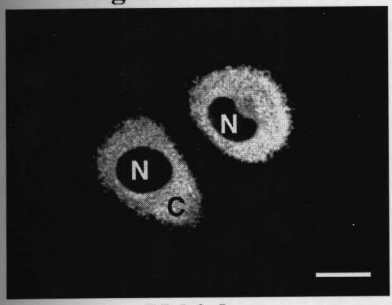
For the confocal analysis, several optical sections (z-series) separated by approximately 0.6 μm were taken for all of the treatments at each of the time points examined. Figure 9 shows five consecutive optical sections from a z-series (8 sections total) of 1,25-$(OH)_2D_3$-treated ROS 17/2.8 cells. As shown in this figure, strong nuclear PKCα fluorescence is visible in all five optical sections.

Figure 7. Optical sections of ROS 17/2.8 cells immunostained with PKCα

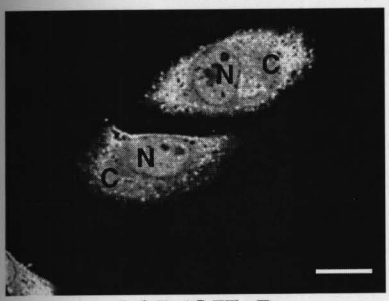
Representative examples of optical sections of immunostained ROS 17/2.8 cells showing PKCα fluorescence distribution after 30 minutes treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Optical sections were obtained using the BioRad MRC-600 confocal microscope. Nuclear PKCα fluorescence can be seen in 1,25-$(OH)_2D_3$-treated cells but not in vehicle-treated cells. Negative control: rabbit-IgG. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm.



Negative Control



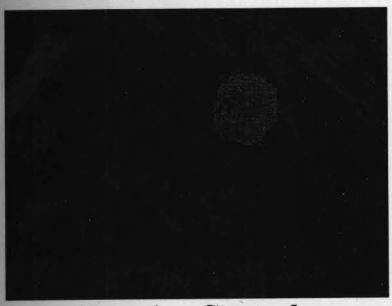
Vehicle



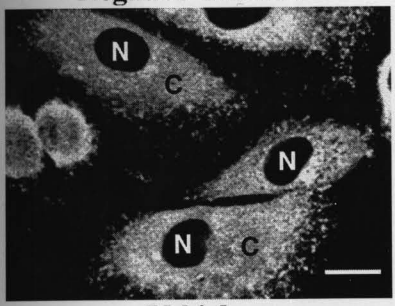
1,25-$(OH)_2D_3$

Figure 8. Optical sections of ROS 17/2.8 cells immunostained with PKCα

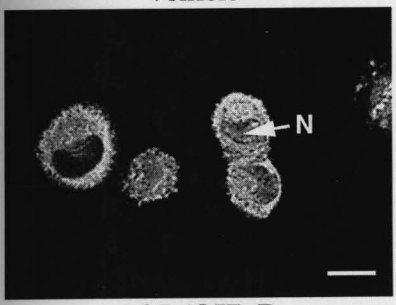
Representative examples of optical sections of immunostained ROS 17/2.8 cells showing PKCα fluorescence distribution after 1 hour treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Optical sections were obtained using the BioRad MRC-600 confocal microscope. Nuclear PKCα fluorescence can be seen in 1,25-$(OH)_2D_3$-treated cells. In control-treated cells PKCα fluorescence is mostly concentrated in the cytosol. Negative control: rabbit-IgG. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm.



Negative Control



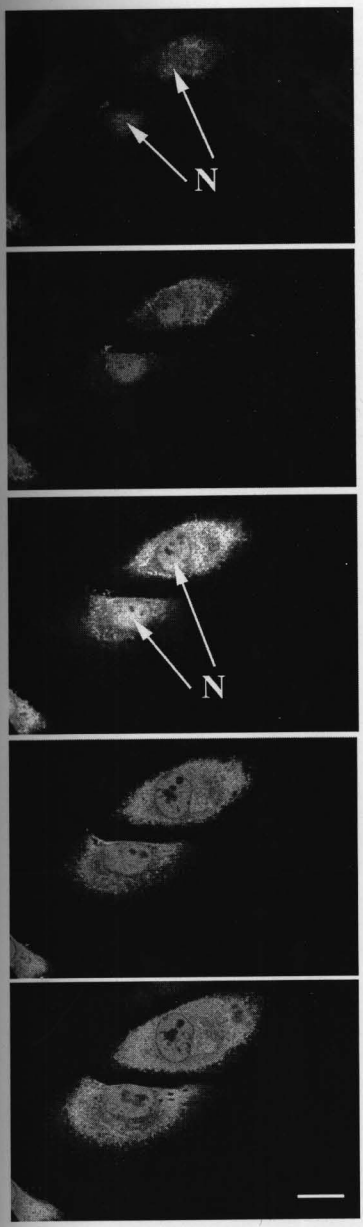
Vehicle



1,25-$(OH)_2D_3$

Figure 9. Optical z-series section of ROS 17/2.8 cells immunostained with PKCα

This figure shows five consecutive optical sections (separated by approximately 0.6 μm) from an eight sections z-series of ROS 17/2.8 cells treated with 20 nM 1,25-$(OH)_2D_3$ for 30 minutes. Optical sections were obtained using the BioRad MRC-600 confocal microscope. Note that nuclear PKCα fluorescence is visible in all five optical sections. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm.


N
N

Effect of 1,25-$(OH)_2D_3$ on PKCα fluorescence at 24 hours

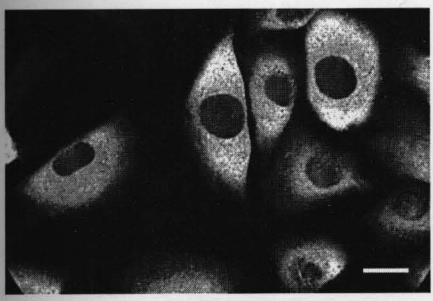
Figure 10 shows optical sections of immunostained ROS 17/2.8 cells at 24 hours treatment. As shown in the figure, the PKCα signal in both control- and 1,25-$(OH)_2D_3$-treated cells was mostly detected in the cytosol. No strong PKCα fluorescence signal was detected in the nuclei in 1,25-$(OH)_2D_3$-treated cells.

PMA increased PKCα fluorescence around the nuclei

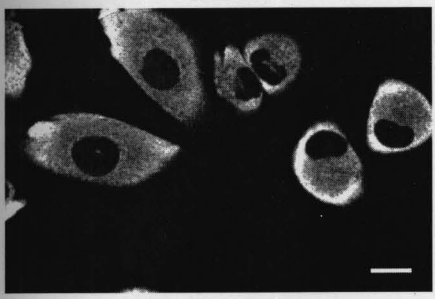
Figure 11 represents confocal images of ROS 17/2.8 cells treated with 100 nM PMA for 30 minutes and 1 hour. As shown in this figure, treatment with PMA for 30 minutes and 1 hour increased PKCα fluorescence, which was localized around the nuclei. PKCα fluorescence was also observed with PMA treatment as punctated areas associated with the plasma membrane and associated with structures encompassing the nucleus. This pattern was not seen in cells treated with either ethanol or 1,25-$(OH)_2D_3$ at any of the time points examined.

Figure 10. Optical sections of ROS 17/2.8 cells immunostained with PKCα

Representative examples of optical sections of immunostained ROS 17/2.8 cells showing PKCα fluorescence distribution after 24 hours treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Optical sections were obtained using the BioRad MRC-600 confocal microscope. PKCα fluorescence is concentrated in the cytosol in both control- and 1,25-$(OH)_2D_3$-treated cells. Nuclei (N), cytosol (C). *Scale Bar* = 15 μm.



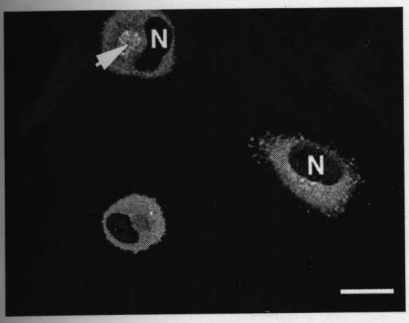
Vehicle



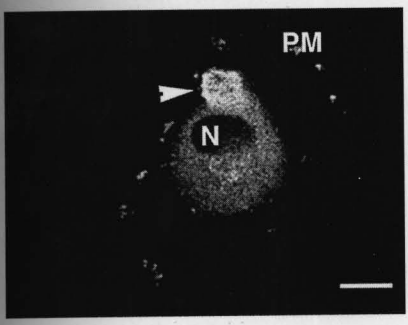
$1,25\text{-}(OH)_2D_3$

Figure 11. Optical section of ROS 17/2.8 cells showing PKCα distribution after treatment with 100 nM PMA

Representative examples of optical sections of immunostained ROS 17/2.8 cells showing PKCα fluorescence distribution at 30 minutes and 1 hour treatment with 100 nM PMA. Optical sections were obtained using the BioRad MRC-600 confocal microscope. As depicted in the sections, PKCα fluorescence is concentrated around the nuclei and as punctated areas associated with the plasma membrane (PM). PKCα fluorescence is also associated with structures encompassing the nucleus (arrow). Nuclei (N), cytosol (C), plasma membrane (PM). *Scale Bar* = 15 μm.



30 Minutes



1 Hour

DISCUSSION

PKC isoenzymes are present predominantly in the cytosol in an inactive state and upon activation they translocate to other cellular compartments. 1,25-$(OH)_2D_3$ has been shown to induce translocation of several PKC isoforms, including PKCα. In Madin Darby bovine kidney (MDBK) cells, treatment with 100 nM 1,25-$(OH)_2D_3$ induced translocation of PKCα to the plasma membrane (Simboli-Campbell et al., 1994). In human promyelocytic leukemia NB4 cells, 200 nM 1,25-$(OH)_2D_3$ induced translocation PKCα to the nuclei and to a particulate fraction (Berry et al., 1996). In human colon cancer-derived Caco-2 cells, 10 nM 1,25-$(OH)_2D_3$ caused translocation of PKCα from the soluble to the particulate fraction (Bissonnette et al., 1994). 1,25-$(OH)_2D_3$-induced translocation of PKCα to the plasma membrane has also been observed in skeletal muscle cells (Capiati et al., 2000). However, to our knowledge, 1,25-$(OH)_2D_3$-induced translocation of PKC isoforms has not been reported in bone cells.

In chapter 2 we reported that 1,25-$(OH)_2D_3$ increased PKCα, but not PKCε, βI or ζ, levels in the nuclei in a time-dependent manner. However, we were not able to certainly detect a decrease in cytosolic PKCα levels by 1,25-$(OH)_2D_3$ treatment, as studied by immunoblotting. Experiments presented in this chapter were designed to study by immunofluorescence (epi-fluorescence and confocal microscopy) the subcellular distribution of PKCα in ROS 17/2.8 cells after treatment with 20 nM 1,25-$(OH)_2D_3$. Here we report that, in accord with the immunoblotting data, 1,25-$(OH)_2D_3$ increased PKCα fluorescence in the nuclear in a time-dependent manner. Moreover, this increase in nuclear PKCα signal was accompanied by a decrease in cytosolic PKCα levels, indicating translocation.

The PKC signaling pathway has been implicated in the regulation of expression of several genes, including: the transcriptional activation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes (Auwerx et al., 1989), down-regulation of the β1-adrenergic receptor gene (Li et al., 1998), and the posttranscriptional regulation of the H-ferritin, GAP-43 and ribonucleotide reductase R1 genes (Pang et al., 1996, Perrone-Bizzozero et al., 1993, Chen et al., 1994). Moreover, previous studies have implicated PKC signaling pathway in the 1,25-$(OH)_2D_3$-mediated regulation of the osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). In this chapter we report that 1,25-$(OH)_2D_3$ translocates PKCα form the cytosol into the nuclei, suggesting a role of PKCα in 1,25-$(OH)_2D_3$-mediated nuclear events (i.e. osteocalcin regulation). On the next chapter we present evidence for a role of PKCα in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression.

originate from the light emission coming form above and below the focal plane. Confocal microscopy was employed to verify the PKCα nuclear signal detected by epi-fluorescence. We corroborated that the PKCα signal detected by epi-fluorescence after 1,25-$(OH)_2D_3$ treatment truly reflected PKCα nuclear fluorescence.

PMA, a known PKCα activator (Nishizuka, 1995), was used in our experiments as a positive control. In accord with the immunoblotting data, treatment with 100 nM PMA increased PKCα fluorescence in the plasma membrane. However, PKCα immunofluorescence in PMA-treated cells concentrated in areas surrounding the nuclei instead of intranuclear sites. Translocation of PKC isoforms to other cellular compartments besides the nuclei and plasma membrane has been reported, including: golgi (Goodnight et al., 1995, Prestle et al., 1996, Westermann et al., 1996), cytoskeleton (Kiley and Parker, 1995, Goodnight et al., 1995), perinucleus (Disatnik et al., 1994, Kiley and Parker, 1995), and endoplasmic reticulum (Chida et al., 1994, Goodnight et al., 1995). Cellular organelles like the endoplasmic reticulum and Golgi apparatus are either structurally connected to the outer membrane of the nuclear envelope or located near the nucleus. Therefore, association of PKCα with those structures may explain why by immunoblotting, but not by immunofluorescence, we detected an increase in PKCα levels in the nuclei with PMA treatment. Nevertheless, immunofluorescence microscopy revealed differences in the regulation of PKCα by 1,25-$(OH)_2D_3$ and PMA. While 1,25-$(OH)_2D_3$ increased intranuclear PKCα, PMA increased PKCα that appears to be associated with the perinucleus and/or with structures encompassing the nuclei.

The PKC signaling pathway has been implicated in the regulation of expression of several genes, including: the transcriptional activation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes (Auwerx et al., 1989), down-regulation of the β1-adrenergic receptor gene (Li et al., 1998), and the posttranscriptional regulation of the H-ferritin, GAP-43 and ribonucleotide reductase R1 genes (Pang et al., 1996, Perrone-Bizzozero et al., 1993, Chen et al., 1994). Moreover, previous studies have implicated PKC signaling pathway in the 1,25-$(OH)_2D_3$-mediated regulation of the osteocalcin gene expression (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). In this chapter we report that 1,25-$(OH)_2D_3$ translocates PKCα form the cytosol into the nuclei, suggesting a role of PKCα in 1,25-$(OH)_2D_3$-mediated nuclear events (i.e. osteocalcin regulation). On the next chapter we present evidence for a role of PKCα in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression.

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Chapter IV

The effect of protein kinase Cα overexpression on osteocalcin gene expression in rat osteoblast cells (ROS 17/2.8)

ABSTRACT

We studied the role of PKCα in osteocalcin regulation since 1,25-$(OH)_2D_3$ was shown to alter PKCα subcellular localization in ROS 17/2.8 cells (chapters 2 and 3). We determined the effect of PKCα overexpression in steady state and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels in ROS 17/2.8 cells. Here we report that overexpression of PKCα blocked the 1,25-$(OH)_2D_3$-induced osteocalcin mRNA expression, without affecting basal osteocalcin levels. To our knowledge, this is the first time evidence is presented for a role of a particular PKC isoform in 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression.

INTRODUCTION

$1,25\text{-}(OH)_2D_3$, the active form of vitamin D, controls calcium and phosphorous homeostasis by acting through intracellular receptors and regulating gene expression in target tissues such as the intestine, kidney and bone (Darwish and DeLuca, 1993). While the actions of $1,25\text{-}(OH)_2D_3$ in the intestine are well characterized, the mechanism by which $1,25\text{-}(OH)_2D_3$ mediates mineral homeostasis in bone is still not well understood. Bone turnover is mainly regulated by osteoblasts and osteoclasts. While osteoclasts are involved in bone resorption, the osteoblasts main function is to synthesize new bone (Marks Jr. and Hermey, 1996). $1,25\text{-}(OH)_2D_3$ has been shown to enhance osteoclastic activity, but receptors for $1,25\text{-}(OH)_2D_3$ have not been identified in these bone-resorbing cells. It has been proposed that under the influence of $1,25\text{-}(OH)_2D_3$, osteoblasts secrete a factor(s) that increases osteoclastic bone resorptive activity (McSheehy and Chambers, 1987). Osteocalcin, the major noncollagenous protein present in the extracellular matrix and exclusively produced by osteoblasts, has been proposed as a possible candidate (Lian et al., 1984, Glowacki and Lian, 1987).

Evidence against the role of osteocalcin in bone resorption has also been presented. Vitamin K deficient rats, whose bone osteocalcin levels were 2% of normal, showed no abnormalities in bone size and mineralization (Price and Williamson, 1981). In another report, osteocalcin-deficient mice developed a phenotype characterized by a higher bone mass than normal mice (Ducy et al., 1996). However, the "loss-of-function" of osteocalcin did not appear to affect either bone resorption or bone mineralization, suggesting that the function of osteocalcin is to limit bone formation without lessening bone resorption.

Osteocalcin is a relevant model to study bone protein regulation by 1,25-$(OH)_2D_3$ regardless of the function(s) that it mediates. The transcriptional regulation of the osteocalcin gene by 1,25-$(OH)_2D_3$ has been extensively studied (Demay et al., 1989, Breen et al., 1994, Mosavin and Mellon, 1996). The proposed mechanism of action includes binding of the active hormone to its intracellular receptor, followed by binding of the hormone-receptor complex to hormone response elements in the osteocalcin gene with the concomitant activation of transcription. Our laboratory reported that 1,25-$(OH)_2D_3$ also regulates osteocalcin gene expression at posttranscriptional level (Mosavin and Mellon, 1996). However, the molecular mechanism(s) by which 1,25-$(OH)_2D_3$ regulates the transcriptional activation and stabilization of osteocalcin mRNA are still not well understood.

Over the last few years various studies have implicated the involvement of several kinases in the actions of 1,25-$(OH)_2D_3$. Up-regulation, down-regulation and phosphorylation of the vitamin D receptor by PKA and PKC have been reported (Darwish et al., 1993, Darwish and DeLuca, 1993, van Leeuwen et al., 1990, Hsieh et al., 1993, Krishnan and Feldman, 1991). PKC has also been implicated in the actions of 1,25-$(OH)_2D_3$ in several cell types, including bone (van Leeuwen et al., 1992, Bissonnette et al., 1994, Simboli-Campbell et al., 1994, Desai et al., 1995, Berry et al., 1996, Simpson et al., 1998, Capiati et al., 1999).

Evidence for the functional involvement of PKC in the actions 1,25-$(OH)_2D_3$ in bone with respect to osteocalcin regulation has been reported. Our laboratory and others reported that treatment of ROS 17/2.8 cells with several PKC inhibitors abolished the 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis (van Leeuwen et al., 1992, Mosavin, 1996). In another study,

the PKC inhibitor staurosporine was shown to inhibit the 1,25-$(OH)_2D_3$-mediated induction of osteocalcin gene transcription (Desai et al., 1995). Moreover, our laboratory demonstrated that inhibition of PKC by the potent inhibitor bisindolylmaleimide blocked the 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA (Mosavin, 1996).

The data presented in chapters 2 and 3 demonstrated that 1,25-$(OH)_2D_3$ specifically translocated PKCα to the nuclei, suggesting a role for this isoform in 1,25-$(OH)_2D_3$-mediated nuclear actions. In the past, PKC activators and inhibitors have been used to study PKC involvement in signaling transduction and gene regulation. The main disadvantage with this approach is that most of those compounds show low specificity for PKC compared to other kinases. Moreover, they have little PKC isozyme selectivity. Therefore, to determined the role of PKCα in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression, PKCα was overexpressed in ROS 17/2.8 cells. Here we report that overexpression of PKCα blocked the 1,25-$(OH)_2D_3$-induced osteocalcin mRNA expression. To our knowledge, this is the first time evidence is presented for a role of a PKC isoform in the regulation of osteocalcin gene expression.

MATERIALS AND METHODS

Materials 1,25-$(OH)_2D_3$ was kindly provided by Dr. Hector F. DeLuca (University of Wisconsin-Madison). LipofectAMINE™ 2000 Reagent was obtained from GibcoBRL (Gaithersburg, Maryland). pRSVneo and pRSVPKCα plasmids were kindly provided by Dr. Ajit K. Verma (University of Wisconsin-Madison). The pRSVneo plasmid (control) contains the neomycin resistant gene and the pRSVPKCα plasmid contains the complete PKCα coding

sequence (Tseng et al., 1994). This vector uses the Rouss sarcoma virus long terminal repeat to drive the expression of PKCα. Rabbit polyclonal IgG antibody that recognizes PKCα and peroxidase-conjugated anti-rabbit IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, California). cPKCα antibody was raised against a peptide mapping at the carboxy terminus of cPKCα of human origin. cPKCα antibody recognizes PKCα of mouse, rat and human origin.

Cell culture ROS 17/2.8 cells were maintained in Ham's F-12 (1:5) culture medium supplemented with 5% (vol/vol) defined fetal bovine serum (Hyclone, Utah), penicillin (100 u/ml), and streptomycin (0.1 mg/ml). Cells were propagated continuously at 37^0C under a humidified 5% CO_2: 95% air mixture. For the transfection experiments, cells were plated at a density of 1.8×10^4 cells/cm^2 on 55 cm^2 petri dishes and allowed to adhere overnight. When the cells reached 90% confluence they were transfected with control plasmid (pRSVneo) or PKCα-containing plasmid (pRSVPKCα). Experiments were carried out on cells between the 5th and 30th passage.

Transfection Two days before transfection, cells were trypsinized and plated at a density of 1.8×10^4 cells/cm^2 on 55 cm^2 petri dishes, so that they were about 90% confluent on the day of transfection. Cells were growth in normal media containing serum but without antibiotics. Transfections were performed as described by the manufactured with some modifications (GibcoBRL). For each petri dish, 55 µl of LipofectAMINE™ 2000 (LF2000) Reagent were diluted in 1.4 ml of serum-free media (without antibiotics) and incubated for 5 minutes. 10 µg of pRSVneo or pRSVPKCα cDNA were diluted in 1.4 ml serum-free media (without antibiotics),

for each petri dish. The diluted cDNA and the diluted LF2000 Reagent were combined and incubated at room temperature for 20 minutes. Cells were fed with normal growth media (without antibiotics) and the DNA-LF2000 complexes were added to each plate. The cells were incubated at 37^0C in CO_2 incubator for 24 hours. Twenty-four hours after transfection cells were either tripsinised and replated in normal media containing antibiotics at a density of 1.1×10^4 cells/cm^2 in 55 cm^2 petri dishes, or cell homogenates were prepared and western blot analysis was performed to determine PKCα overexpression. When the cells reached 50-70% confluence (30 hours) they were treated with either ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Twenty-four hours after treatment cells were assayed for PKCα protein expression (nuclear fraction) and/or osteocalcin mRNA levels. Both cell density and confluence were kept constant between experiments.

Preparation of cell homogenates All the steps were performed at 4^0C. The cells were washed and collected in a chilled TEDK buffer solution [10 mM Tris-HCl (pH 7.2), 0.3 M KCl, 1 mM EDTA, and 5 mM dithiothreitol (DTT)]. The cells were centrifuged at 500 x g for 10 minutes. The cell pellet was suspended in 1 ml of TEDK, containing 1% protease cocktail inhibitor solution (Sigma, St. Louis, Missouri), and homogenized in a Dounce homogenizer (40 strokes). Cell homogenates were used for western blot analysis.

Preparation of nuclear fraction Nuclear fractions were prepared as described before with some modifications (Delude et al., 1994). All the steps were performed at 4^0C. Cells were washed and collected in a chilled 1X PBS buffer solution. The cell suspension was centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 600 µl of Buffer A [10 mM Tris

(pH 7.8), 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.3 M sucrose, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. Nonidet P-40 was added to 0.6% (vol/vol) and the tubes were vortexed at setting 10 with a Vortex mixer (Spectra-Physics, California) for 20 seconds. Nuclei were harvested by centrifugation of the samples at 7,200 x g for 30 seconds. The pellet (nuclei) was resuspended in 300 µl of Buffer B [20 mM Tris (pH 7.8), 5 mM $MgCl_2$, 320 mM KCl, 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. The samples were centrifuged at 13,500 x g for 15 minutes. The resulting supernatant (nuclear extract) was collected.

Immunoblotting For immunoblotting, samples (cell homogenates or nuclear fractions) were diluted 1:1 (vol/vol) in 2X sample buffer [12 mM Tris, 1.5 mM EDTA, 20 mM DTT, 2% SDS, 20% glycerol, and 0.2% bromophenol blue] and solubilized by boiling for 5 minutes. The samples were electrophoresed in a 12.5% SDS-polyacrylamide minigel for 90 minutes at 160 V in 1X running buffer (250 mM Tris, 10 M glycine, and 0.01% SDS). The proteins were transferred electrophoretically to a PVDF membrane at 0.60 A (constant voltage) for 2 hours at 4^0C in 1X transblot buffer (250 mM Tris, 10 M glycine). The blots were blocked with 2.5% non-fat dry milk in TBST (1 mM Tris, 15 mM NaCl and 0.05% Tween) overnight at 4^0C. Following blocking, the blots were probed with polyclonal antibody for PKCα for 1 hour at 37^0C. The blots were washed three times for 5 minutes at 37^0C in TBST and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase for 50 minutes at 37^0C. The blots were washed subsequently 2 times for 15 minutes at 37^0C in TBST.

RNA isolation Total RNA was isolated using the guanidinium-pheno-bromochloropropane method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987, Chomczynski and Sacchi, 1995). Briefly, approximately 2 x 10^6 cells were suspended in 200 µl of homogenization buffer (4 M guanidinium thiocynate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol). Addition of 20 µl of 2 M sodium acetate, 200 µl water-saturated phenol, and 40 µl bromochloropropane followed. The tubes were vortexed for 10 seconds and placed on ice for 15 minutes. The tubes were centrifuged at 10,000 x g for 20 minutes at 4^0C and the upper phase, containing the RNA, was transferred to another tube. RNA was precipitated by incubating with isopropanol (1:1) overnight at -20^0C. The RNA was pelleted by centrifugation at 10,000 x g for 20 minutes at 4^0C. The pellet was redissolved in 50 µl homogenization buffer, and 50 µl of isopropanol was added to the tubes. The RNA was reprecipitated overnight at -20^0C. The RNA was pelleted by centrifugation at 10,000 x g for 15 minutes at 4^0C, washed with 70% ethanol and resuspended with diethyl pyrocarbonate (DEPC)-treated water.

Northern blot analysis Total RNA was electrophoresed in a 1.2% agarose/formaldehyde gel. After electrophoresis RNA was transferred overnight to Hybond-N nylon membrane (Amersham, Arlington Heights, IL) using 20X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.4). RNA was crosslinked to the membrane by UV irradiation using a Stratalinker (Stratagene, La Jolla, CA). The blots were prehybridized for 1 hour at 42^0C in a hybridization buffer (ULTRAhyb) obtained from Ambion (Austin, Texas). The blots were hybridized to osteocalcin cDNA probe overnight at 42^0C using 10 x 10^6 cpm of probe labeled by nick translation (Promega, Madison, Wisconsin) with [α-^{32}P]dCTP. Following hybridization, the

blots were washed twice for 10 minutes at room temperature with 2X SSPE/0.1% SDS, once with 1X SSPE/0.1% SDS for 10 minutes at 42^{0}C and once with 0.1X SSPE/0.1%SDS for 10 minutes at 42^{0}C. The blots were then exposed to a Molecular Dynamics PhosphoImager screen (Molecular Dynamics, Sunny Vale, California).

Detection and data analysis PKCα expression on western blots was detected using the LumiGlo chemiluminescent substrate kit (Kirkegaard & Perry Laboratories, Maryland) and blots were exposed to x-ray film (RX-U, FujiFilm) with Quanta III intensifying screens. Bands were quantified by using the Molecular Dynamics 300 A Computing Densitometer (Sunnyvale, California). The range of the linearity of PKCα density measurements was calculated to be between 5 μg to 50 μg of total protein. To decrease variability in PKCα density measurements between experiments the amount of total protein electrophoresed and the exposure time of blots to x-ray films were kept constant. PKCα levels were expressed relative to control. Statistical comparison were made by using *t*-test (Statistica, StatSoft) for independent sample means at 95% confidence level. The data is expressed as the mean ± the standard error of the mean.

For northern blot analysis the blots were exposed to a Molecular Dynamics PhosphoImager screen (Sunny Vale, California). The PhosphoImager screen was scanned by using the PhosphoImager™ SI from Molecular Dynamics and the bands were quantified by using ImageQuant software (Molecular Dynamics, Sunny Vale, California). The 18S ribosomal band was quantified by using the Molecular Dynamics 300 A Computing Densitometer (Sunnyvale, California). Osteocalcin mRNA was corrected to the 18S ribosomal band and expressed relative to control. Statistical comparisons were made by using *t*-test for independent sample means or

by one way analysis of variance (ANOVA) (Statistica, StatSoft). The data is expressed as the mean ± the standard error of the mean. $p < 0.05$ was considered statistically significant.

RESULTS

Overexpression of PKCα in ROS 17/2.8 cells

The PKC inhibitors studies suggest that PKC is involved in the regulation of osteocalcin gene expression. The data presented in chapter 2 and 3 indicate that 1,25-$(OH)_2D_3$ regulates PKCα subcellular localization. Therefore, we determined the role of PKCα in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression.

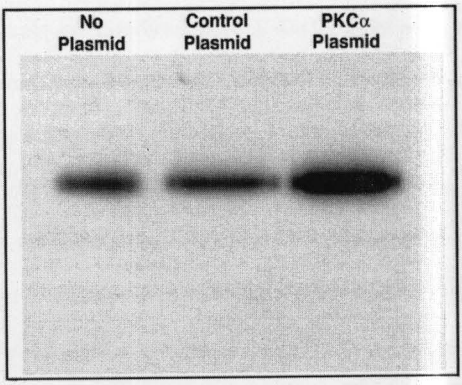
We overexpressed PKCα in ROS 17/2.8 cells. Three different transfecting agents were used for the overexpression experiments. Two of them, LipofectAMINE and LipofectAMINE Plus, either failed to transfect the plasmid or resulted in high cell dead (over 60%). On the other hand, transfection of the PKCα-containing plasmid (pRSVPKCα) with LipofectAMINE 2000 resulted in over 4-fold increase in PKCα levels, and low cell dead (40%) when compared to the other transfecting agents.

For these experiments cells were either transfected with control plasmid (pRSVneo) or PKCα-containing plasmid (pRSVPKCα), or not transfected (no plasmid). Cell homogenates were prepared and PKCα expression was determined by western blotting as described under Materials and Methods. The levels of PKCα in transfected cells were compared to the non-transfected group (no plasmid). The results are summarized in figure 1. Transfection with control plasmid

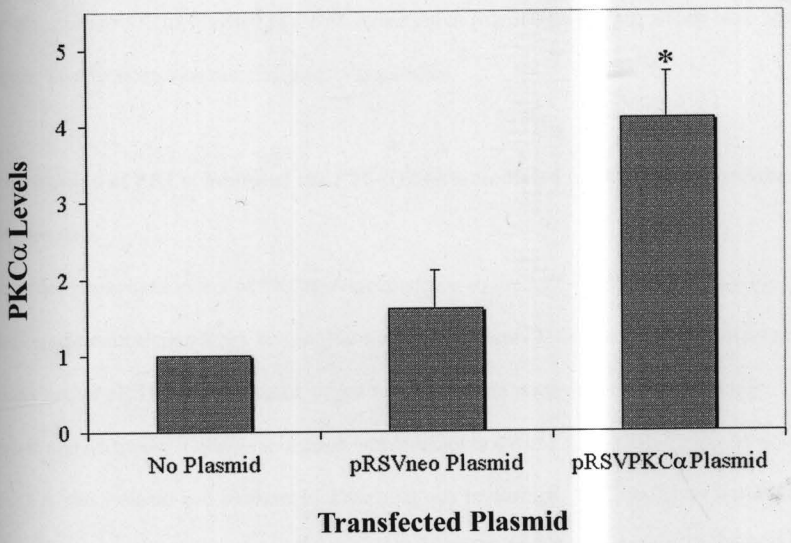
Figure 1. PKCα overexpression in ROS 17/2.8 cells

Panel A: Example of immunoblot for the expression of PKCα levels in ROS 17/2.8 cells. Cells were transfected with either control plasmid (pRSVneo) or PKCα-containing plasmid (pRSVPKCα) as described under Materials and Methods. Cells were collected in a chilled TEDK buffer solution and cell homogenates were prepared. 20 μg of total protein were electrophoresed on a 12.5% SDS-polyacrylamide minigel and western blotting was performed. A 1:2000 dilution of PKCα polyclonal antibody and a 1:8000 dilution of anti-rabbit secondary antibody conjugated to HRP were used. PKCα expression was detected using ECL detection kit followed by autoradiography. **Panel B**: Densitometric analysis of PKCα levels in ROS 17/2.8 cells after transfection. PKCα levels are expressed relative to control (no plasmid). Statistical comparisons were made by using *t*-test for independent sample means. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control (no plasmid)**

A.


No Plasmid
Control Plasmid
PKCα Plasmid

B.


PKCα Levels
0
1
2
3
4
5
*
No Plasmid
pRSVneo Plasmid
pRSVPKCα Plasmid
Transfected Plasmid

resulted in a 1.6-fold increase in PKCα levels compared to PKCα levels in non-transfected cells. However, this was not statistically significant. On the other hand, transfection with the pRSVPKCα plasmid resulted in a 4.1-fold increase in PKCα levels. This increase was statistically significant ($p < 0.05$).

PKCα Overexpression did not alter osteocalcin mRNA steady state levels

We determined the effect of PKCα overexpression on osteocalcin mRNA steady state levels. The results are summarized in figure 2. Transfection of ROS 17/2.8 cells with the pRSVneo plasmid resulted in no change in osteocalcin mRNA levels compared to non-transfected cells. Overexpression of PKCα resulted in a 10% reduction in osteocalcin mRNA steady state levels. However, this decrease was not statistically significant.

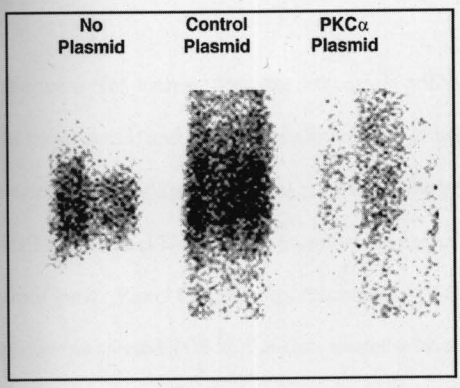
Overexpression of PKCα inhibited the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA levels.

We then determined the effect of PKCα overexpression in the 1,25-$(OH)_2D_3$-mediated up regulation of osteocalcin mRNA levels. For these experiments cells were either transfected with the pRSVneo or pRSVPKCα plasmid, or not transfected (no plasmid), as described under Materials and Methods. Cells were treated with ethanol or 20 nM 1,25-$(OH)_2D_3$ for 24 hours. Total RNA was isolated and northern blot analysis was performed. The results are summarized in figure 3. As expected, treatment of non-transfected ROS 17/2.8 cells with 1,25-$(OH)_2D_3$ increased osteocalcin mRNA levels by 9.3-fold as compared to vehicle-treated cells (no plasmid). As determined previously, transfection of ROS 17/2.8 cells with the pRSVneo plasmid

Figure 2. Effect of PKCα overexpression on osteocalcin mRNA steady state levels

Panel A: Northern blot analysis showing osteocalcin mRNA levels in non-transfected ROS 17/2.8 cells (no plasmid) and in cells transfected with either control plasmid (pRSVneo) or PKCα-containing plasmid (pRSVPKCα). **Panel B**: Densitometric analysis of osteocalcin mRNA levels in ROS 17/2.8 cells after transfection. **Panel C**: Ethidium bromide staining of the blot depicted in panel A showing 18S ribosomal band. Osteocalcin mRNA was corrected to 18S ribosomal band and expressed relative to control (no plasmid). Statistical comparisons were made by using *t*-test for independent sample means. The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. ***p < 0.05 vs. control (no plasmid)**

A.


No Plasmid
Control Plasmid
PKCα Plasmid

C.


18S

B.

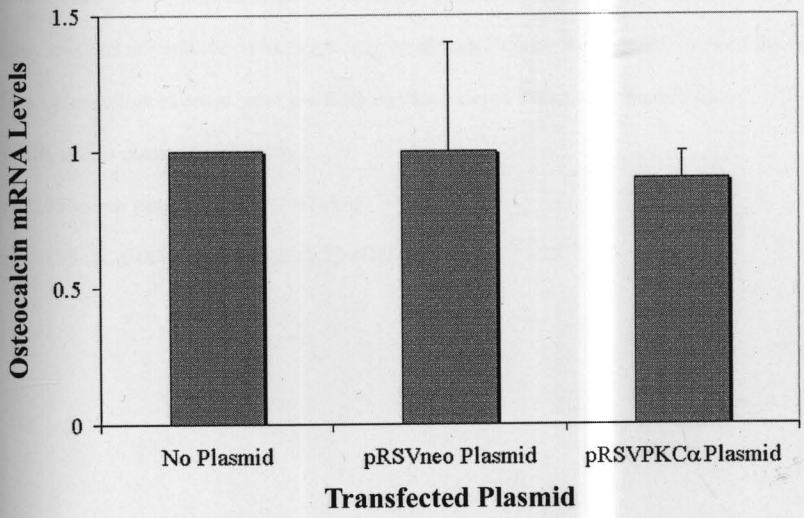

Osteocalcin mRNA Levels
1.5
1
0.5
0
No Plasmid
pRSVneo Plasmid
pRSVPKCα Plasmid
Transfected Plasmid

Figure 3. Effect of PKCα overexpression on 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels

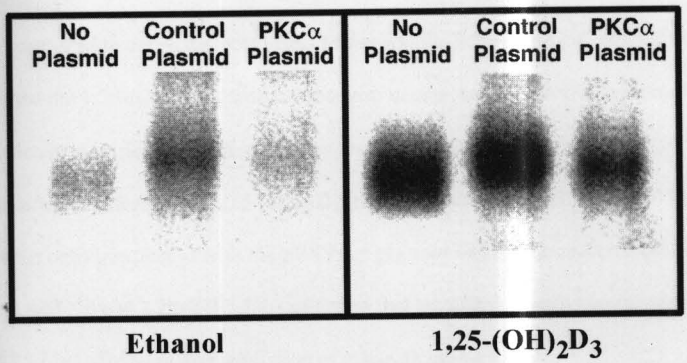
Panel A: Northern blot analysis showing osteocalcin mRNA levels in non-transfected ROS 17/2.8 cells (no plasmid) and in cells transfected with either control plasmid (pRSVneo) or PKCα-containing plasmid (pRSVPKCα) at 24 hours treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. **Panel B**: Ethidium bromide staining of the blot depicted in panel A showing 18S ribosomal band. **Panel C**: Densitometric analysis of osteocalcin mRNA levels in non-transfected and transfected ROS 17/2.8 cells treated with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$ for 24 hours. Osteocalcin mRNA was corrected to 18S ribosomal band and expressed relative to control (no plasmid, ethanol treatment). The statistical significance was assessed by one way analysis of variance (ANOVA). The error bars indicate the standard error of the mean of three independent experiments. **$p < 0.05$** was considered statistically significant.

***$p < 0.05$ vs. no plasmid (ethanol)**

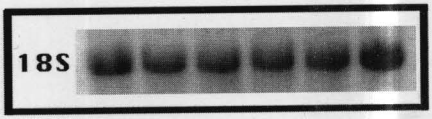
****$p < 0.05$ vs. no plasmid (1,25-$(OH)_2D_3$)**

*****$p < 0.05$ vs. pRSVneo plasmid (1,25-$(OH)_2D_3$)**

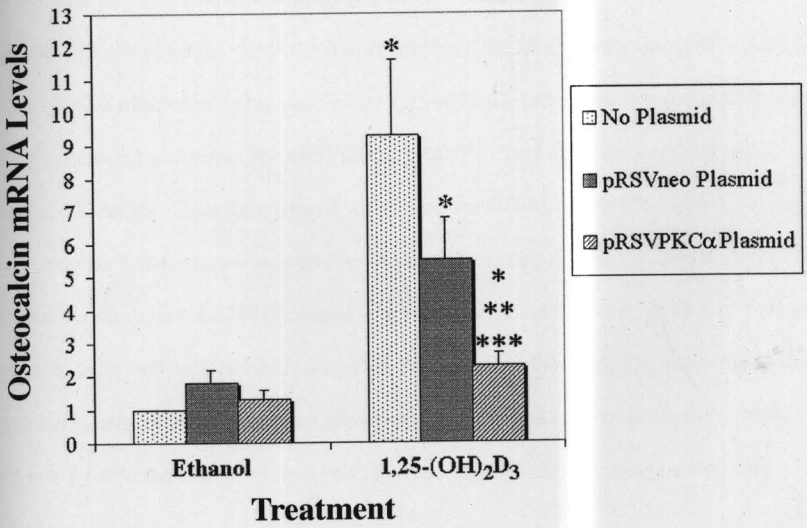
A.


No Plasmid
Control Plasmid
PKCα Plasmid
No Plasmid
Control Plasmid
PKCα Plasmid
Ethanol
1,25-(OH)2D3

B.


18S

C.


Osteocalcin mRNA Levels
13
12
11
10
9
8
7
6
5
4
3
2
1
0
*
*
*
**
***
Ethanol
1,25-(OH)2D3
Treatment
No Plasmid
pRSVneo Plasmid
pRSVPKCα Plasmid

or the pRSVPKCα plasmid resulted in no significant change in osteocalcin mRNA steady state levels as compared to non-transfected cells (ethanol). On the other hand, overexpression of PKCα diminished the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA levels by 75%. This was statistically significant as determined by one way analysis of variance (ANOVA). No statistically significant difference in 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels was observed between cells transfected with the pRSVneo plasmid and non-transfected cells after 24 hours treatment with 20 nM 1,25-$(OH)_2D_3$, indicating that transfection with control-plasmid had no effect on 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin mRNA.

Effect of 20 nM 1,25-$(OH)_2D_3$ treatment on PKCα nuclear levels in ROS 17/2.8 cells overexpressing PKCα

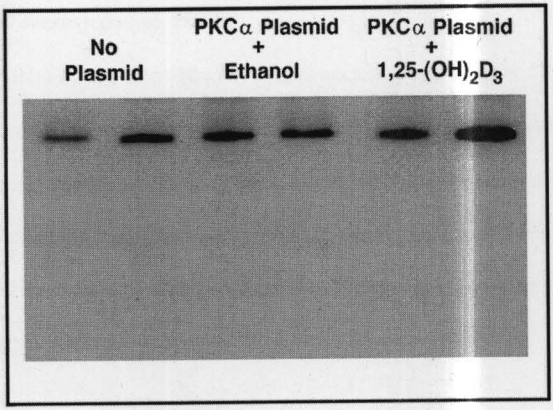
We also studied 1,25-$(OH)_2D_3$-mediated translocation of PKCα in ROS 17/2.8 cells transfected with the pRSVPKCα plasmid. We wanted to determine if the PKCα overexpressed in ROS 17/2.8 cells would translocate to the nuclei after 1,25-$(OH)_2D_3$ treatment. For these experiments cells were transfected with either the pRSVneo or pRSVPKCα plasmid as described under Materials and Methods. Cells were treated with ethanol or 20 nM 1,25-$(OH)_2D_3$ for 1 hour and 24 hours. Nuclear fractions were prepared and western blot analysis was performed. Figure 4 summarizes the results for the 24 hours treatment experiment. As shown in the figure, treatment of transfected cells with 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by 2.4-fold compared to non-transfected cells. On the other hand, treatment of transfected cells with vehicle (ethanol) resulted in a 1.5-fold increase in nuclear PKCα levels, respectively. This experiment was performed twice.

Figure 5 summarizes the effect of 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ on PKCα levels in the nuclei. As shown in the figure, treatment of transfected cells with 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by 2.8-fold compared to non-transfected cells. This experiment was performed once.

Figure 4. Effect of 24 hours treatment with 20 nM 1,25-$(OH)_2D_3$ on PKCα nuclear levels in ROS 17/2.8 cells overexpressing PKCα

Panel A: Example of immunoblot for the expression of PKCα nuclear levels in ROS 17/2.8 cells after 24 hours treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Nuclear fractions were prepared as described previously. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed. **Panel B**: Densitometric analysis of PKCα nuclear levels in non-transfected and tranfected ROS 17/2.8 cells treated with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$ for 24 hours. PKCα levels are expressed relative to control (no plasmid). The data represent two independent experiments performed in duplicates. The numbers in parenthesis represent the lower and higher levels of two determinations.

A.

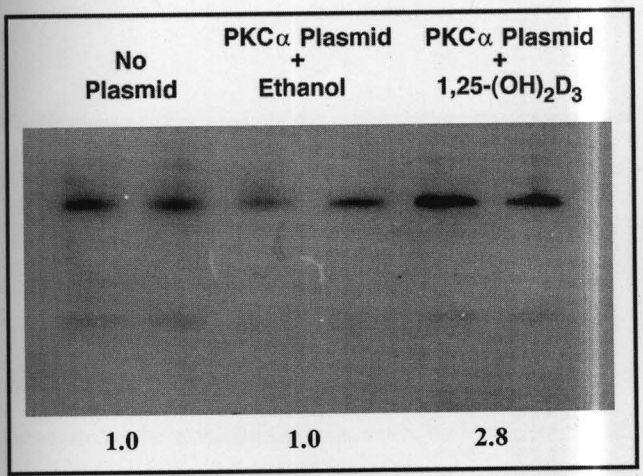


B.

TRANSFECTED PLASMID	TREATMENT	FOLD-INDUCTION
No Plasmid	No treatment	1.0 (0.8, 1.2)
pRSVPKCα	ETHANOL	1.5 (0.9, 2.3)
pRSVPKCα	$1,25\text{-}(OH)_2D_3$	2.4 (1.4, 3.1)

Figure 5. Effect of 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ on PKCα nuclear levels in ROS 17/2.8 cells overexpressing PKCα

Example of immunoblot for the expression of PKCα nuclear levels in ROS 17/2.8 cells after 1 hour treatment with ethanol (as vehicle) or 20 nM 1,25-$(OH)_2D_3$. Nuclear fractions were prepared as described previously. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blotting was performed. This experiment was performed once. The values at the bottom of the figure represent PKCα levels as expressed relative to control (no plasmid).


No Plasmid
PKCα Plasmid + Ethanol
PKCα Plasmid + 1,25-(OH)2D3
1.0
1.0
2.8

DISCUSSION

The PKC signaling pathway is involved in transduction responses to a vast array of agonists. Besides PKC's role in signaling, it is widely accepted that PKC regulates "long term" cellular processes such as proliferation and differentiation. Many of the physiological functions mediated by PKC involve nuclear events, including regulation of gene expression. For example, PKC is known to phosphorylate RNA Polymerase II (*in vitro*), Lamin B (*in vitro/in vivo*), DNA polymerase α and β (*in vitro*) and several histones (*in vitro*) (Buchner, 1995). PKC has also been implicated in the transcriptional activation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes (Auwerx et al., 1989), down-regulation of the β1-adrenergic receptor gene (Li et al., 1998), and the posttranscriptional regulation of the H-ferritin, GAP-43 and ribonucleotide reductase R1 genes (Pang et al., 1996, Perrone-Bizzozero et al., 1993, Chen et al., 1994).

Evidence for the involvement of PKC in the actions of 1,25-$(OH)_2D_3$ in bone in respect to the regulation of osteocalcin expression has been reported. Van Leeuwen and coworkers studied the involvement of PKC in the osteocalcin gene expression and secretion in ROS 17/2.8 cells. They reported that activation of PKC with 100 nM PMA did not cause an increase in osteocalcin secretion, while only a small increase in osteocalcin synthesis was observed (van Leeuwen et al., 1992). However, PMA did not further change the 1,25-$(OH)_2D_3$-induced up-regulation of osteocalcin synthesis. Our laboratory reported the effect of 24 hours treatment with 1 nM, 10 nM, 50 nM doses of PMA on basal and 1,25-$(OH)_2D_3$ induced osteocalcin mRNA levels in ROS 17/2.8 cells. Treatment with 1 nM PMA for 24 hours caused a small increase in osteocalcin

mRNA steady state levels (Mosavin, 1996). However, no significant change was observed with 10 or 50 nM PMA. On the other hand, in the presence of 1,25-$(OH)_2D_3$ (5 nM), treatment with 50 nM PMA increased the 1,25-$(OH)_2D_3$-mediated up-regulation of osteocalcin mRNA by about 1.8-fold. However, this effect was not observed when cells were treated with 1 and 10 nM PMA in the presence of 1,25-$(OH)_2D_3$. It is important to mention that in both reports ROS 17/2.8 cells were treated with PMA for 24 hours. Down-regulation of PKC isoenzymes by PMA and other phorbol esters has been reported in several cell lines, including bone cells, after prolonged period of treatment (Szallsi et al., 1994, Johnson et al., 1995, Keenan et al., 1995, Sanders and Stern, 1996). Sanders and Stern studied the sensitivity of PKC isoforms to prolonged phorbol ester treatment in several osteoblast cells, including ROS 17/2.8 cells. They reported down-regulation of the expressed conventional and novel PKC isoforms after 6 hours of phorbol 12,13-dibutyrate treatment (Sanders and Stern, 1996).

The effect of PKC inhibition in the regulation of osteocalcin gene expression has also been reported. Van Leeuwen determined that treatment of ROS 17/2.8 cells with 25 μM 1-O-hexadecyl-2-O-methyl-rac-glycerol (AMG) or 25 μM sphingosine inhibited 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis, without affecting basal osteocalcin synthesis (van Leeuwen et al., 1992). In another report by Desai and coworkers, the PKC inhibitor staurosporine (100 nM) inhibited the 1,25-$(OH)_2D_3$-mediated induction of osteocalcin gene transcription (Desai et al., 1995). Our laboratory determined the effect of three PKC inhibitors (bisindolylmaleimide, calphostin C and sphingosine) on basal and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels (Mosavin, 1996). Treatment of ROS 17/2.8 cells with sphingosine (10 μM), alone or in the presence of 1,25-$(OH)_2D_3$ (20 nM), for 24 hours did not alter osteocalcin mRNA levels. On the

other hand, calphostin C (100 nM) and bisindolylmaleimide (5 μM and 20 μM) caused a small decrease in osteocalcin steady state levels. Furthermore, in the presence of 1,25-$(OH)_2D_3$ (20 nM) both inhibitors decreased the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA by over 75%. Bisindolylmaleimide (20 μM) was also reported to blocked the 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA (Mosavin, 1996).

Several discrepancies ensue from these reports. First, it is important to mention the mechanism of action of these inhibitors. Sphingosine and calphostin C interact with the regulatory domain, specifically the conserved region C1 (Hannun et al., 1986, Kobayashi et al., 1989). This region contains cysteine rich-sequences that bind to DAG and the phorbol esters. On the other hand, bisindolylmaleimide interacts with the ATP binding domain of PKC (Toullec et al., 1991), another conserved domain (C3). These conserved regions are also present in other proteins, including protein kinases (Mochly-Rosen and Kauvar, 1998, Ron and Kazanietz, 1999). Therefore, it is possible that other protein kinases were inhibited in these studies. For instance, bisindolylmaleimide is a potent PKC inhibitor with a constant for inhibition of 10 nM (Toullec et al., 1991). However, inhibition of PKA (Ki = 2 μM) has also been reported (Muid et al., 1991). 5 and 20 μM doses of bisindolylmaleimide were used in the studies described above. Therefore, the possibility of PKA inhibition may not be ruled out. Accordingly, while van Leeuwen reported inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis by sphingosine, our laboratory didn't detect changes in osteocalcin mRNA levels with sphingosine treatment.

In chapters 2 and 3 we reported that 1,25-$(OH)_2D_3$ specifically increased PKCα levels in the nuclei in ROS 17/2.8 cells, suggesting a role for this isoform in 1,25-$(OH)_2D_3$-mediated

regulation of osteocalcin synthesis. As discussed above, PKC activators and inhibitors were used in the past to study the involvement of PKC in the regulation of osteocalcin expression. However, the data is fraught with discrepancies. To study the role of PKCα in the regulation of osteocalcin expression we took a different approach. We overexpressed PKCα in ROS 17/2.8 cells, and determined the effect of PKCα overexpression on steady state and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels.

First, we successfully overexpressed PKCα in ROS 17/2.8 cells. Increases in PKCα levels by 4- to 5-fold were observed even four days after transfection. We then tested if the PKCα overexpressed was also regulated by 1,25-$(OH)_2D_3$. We determined that 1 hour and 24 hours treatment with 20 nM 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by 2.4- and 2.8, respectively. Finally, we studied the effect of PKCα overexpression in the regulation of osteocalcin expression. We found that overexpression of PKCα inhibited by over 75% the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA levels, without affecting basal osteocalcin levels.

These results contradict the PKC inhibitors data discussed above. Several possible explanations are discussed therein. First, in the PKC inhibitors experiments there exist the possibility that besides PKC other kinases were also inhibited. This may explained why a reduction in 1,25-$(OH)_2D_3$-induced osteocalcin levels was observed with the PKC inhibitors. Second, even though we determined that PKCα was the only isoform regulated by 1,25-$(OH)_2D_3$ it may be that other PKC isoform(s) not investigated in our studies is(are) involved in the regulation of osteocalcin expression. For instance, we didn't study PKCβII and ι regulation by 1,25-$(OH)_2D_3$ and these

isoforms are known to be expressed in ROS 17/2.8 cells (Sanders and Stern, 1996). Moreover, opposing effects by PKC isozymes has been reported. Mochly-Rosen and coworkers studied the role of PKCε and PKCδ in contraction rates of cardiac myocytes. They reported that inhibition of PKCε activity inhibited, whereas inhibition of PKCδ enhanced, hormone- and PMA-induced contraction rate in these cells (Johnson et al., 1996, Csukai and Mochly-Rosen, 1998). In another report, Mischak and coworkers studied the effect of overexpression of PKCδ and PKCε in NIH 3T3 cells. They found that overexpression of PKCδ induced significant changes in morphology and caused the cells to growth more slowly and to a decreased cell density in confluent cells (Mischak et al., 1993). On the other hand, overexpression of PKCε resulted in increased growth rates and higher cell densities, but caused no change in cell morphology. These results taken together suggest that more than one isoform may be implicated in PKC-mediated regulation of a particular cellular function.

The third possibility is that the PKCα overexpressed in ROS 17/2.8 cells was not regulated by 1,25-$(OH)_2D_3$. However, we determined that 1,25-$(OH)_2D_3$ increased nuclear PKCα levels in cells transfected with the PKCα-containing plasmid by 2.4- and 2.8-fold at 1 and 24 hours, respectively. Therefore, 1,25-$(OH)_2D_3$-mediated regulation of PKCα occurred in cells overexpressing this isoform. On the other hand, these PKCα fold-inductions in transfected cells are of the same magnitude as the1,25-$(OH)_2D_3$-mediated fold-increases observed in non transfected ROS 17/2.8 cells, as discussed in chapter II. Therefore, this third possibility should not be ruled out. Finally, it may be that events related with the transfection protocol (e.i. transfecting agent, transfected-plasmid) blocked the 1,25-$(OH)_2D_3$-induced increase in osteocalcin mRNA levels. However, our results indicate differently. First, as shown in figure 2,

transfection with the pRSVneo plasmid (control plasmid) resulted in no significant change in osteocalcin steady state mRNA levels compare to non-transfected cells. Moreover, no significant difference in 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels was observed between cells transfected with the pRSVneo plasmid (control plasmid) and non-transfected cells after 24 hours treatment with 20 nM 1,25-$(OH)_2D_3$.

In this chapter we present evidence that suggest that PKCα is involve in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression. To our knowledge, this is the first report that postulates a role of a particular PKC isoform in the regulation of osteocalcin expression. Nevertheless, our observations do not provide us with irrefutable evidence. To further study PKC's role in osteocalcin regulation, future experiments should include overexpression of other PKC isozymes and the use of antisense oligonucleotides directed to PKCα and/or other isoforms. The effect of protein kinase Cα on 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA should be investigated.

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Chapter V

CONCLUDING REMARKS

Several reports have implicated a protein kinase C (PKC) signaling pathway in 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]-mediated actions in various tissues (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 1999, Simpson et al., 1998). Evidence for the functional involvement of PKC in the actions of 1,25-$(OH)_2D_3$ in bone in respect to osteocalcin regulation has also been reported. It has been shown by our laboratory and others that treatment of ROS 17/2.8 cells with several PKC inhibitors abolished the 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis (van Leeuwen et al., 1992, Mosavin, 1996). In another report, staurosporine (PKC inhibitor) was shown to inhibit 1,25-$(OH)_2D_3$-mediated induction of osteocalcin transcription (Desai et al., 1995). Moreover, our laboratory demonstrated that inhibition of PKC by the potent inhibitor bisindolylmaleimide inhibited the 1,25-$(OH)_2D_3$-mediated stabilizaton of osteocalcin mRNA (Mosavin, 1996).

PKC is a family of serine/threonine kinases that consists of at least 12 isoforms. These isoforms are involved in transduction responses to a vast array of agonists such as neurotransmitters, growth factors and hormones (Dekker and Parker, 1994, Nishizuka, 1995). PKCs are mostly present in the cytoplasm in an inactive state and upon activation they translocate to the plasma membrane where they regulate the catalytic activity of target proteins through phophorylation. However, translocation to other cellular compartments, including the nuclei, has also been reported (Chida et al., 1994, Disatnik et al., 1994, Buchner, 1995, Goodnight et al., 1995, Kiley and Parker, 1995, Prestle et al., 1996, Westermann et al., 1996). Regulation of PKC expression and activity by 1,25-$(OH)_2D_3$ has been studied in several cell lines (Obeid et al., 1990, Solomon et al., 1991, Simboli-Campbell et al., 1994, Berry et al., 1996, Capiati et al., 1999). However, regulation of PKC isoforms by 1,25-$(OH)_2D_3$ in bone cells was not previously reported.

To establish the relevance of PKC to the 1,25-$(OH)_2D_3$ intracellular signaling pathway we determined the expression and regulation of PKC isoforms by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells by immunoblotting, immunofluorescence and RT-PCR. We determined that 1,25-$(OH)_2D_3$ increased PKCα, but not βI, ζ and ε, levels in the nuclei in a time-dependent manner. This increase was accompanied by a concomitant decrease in cytosolic PKCα levels, as determined by immunofluorescence. We also determined that 1,25-$(OH)_2D_3$ did not significantly altered PKCα mRNA levels. Moreover, inhibition of transcription with actinomycin D did not alter the 1,25-$(OH)_2D_3$-mediated induction of nuclear PKCα protein levels. Together this data indicates that 1,25-$(OH)_2D_3$ increased PKCα nuclear levels mainly by translocation and gene expression does not appear to play a significant role. Finally, we studied the effect of PKCα overexpression in steady state and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels since 1,25-$(OH)_2D_3$ specifically regulated this isoform. We determined that PKCα overexpression blocked the 1,25-$(OH)_2D_3$-induced osteocalcin mRNA expression, without affecting basal osteocalcin levels.

Mechanism(s) for 1,25-$(OH)_2D_3$-mediated activation of PKCα

Several possible mechanisms by which 1,25-$(OH)_2D_3$ may up-regulate PKCα nuclear levels are discussed therein. First, PKCα, a conventional isoform, is regulated by Ca^{2+}, diacylglycerol (DAG) and phorbol esters. 1,25-$(OH)_2D_3$ has been shown to regulate $[Ca^{2+}]_i$ in several cell lines, including bone (Nemere et al., 1984, Nemere and Norman, 1987, Baran and Milne, 1986, Lieberherr, 1987, Civitelli et al., 1990, Baran et al., 1991, Capiati et al., 2000). In some instances the increase in intracellular Ca^{2+} levels have been correlated with activation of PKC. For example, Capiati et al. reported that 1,25-$(OH)_2D_3$ induced a rapid (30-60 seconds) and sustained (> 5 minutes) increase in intracellular Ca^{2+} levels in skeletal muscle cells (Capiati et

al., 2000). This increase was correlated to translocation of PKCα from the cytosol to the particulate fraction after 5 minutes treatment with 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ has also been reported to modulate dihydropyridine-sensitive Ca^{2+} currents in ROS 17/2.8 cells (Caffrey and Farach-Carson, 1989). Moreover, 1,25-$(OH)_2D_3$ was reported to increase nuclear Ca^{2+} levels in isolated rat liver nuclei (Baran et al., 1989). Therefore, 1,25-$(OH)_2D_3$ may induce translocation of PKCα form the cytosol to the nuclei by regulating intracellular Ca^{2+} levels.

Second, generation of DAG may be another mechanism by which 1,25-$(OH)_2D_3$ can regulate PKCα subcellular distribution. DAG is generated upon stimulation of cell surface receptors by the hydrolysis of phosphatidylinositol 4,5-bisphosphonate (PIP_2) by phospholipase C (PLC). This first phase of DAG generation disappears promptly (seconds). A second phase of DAG generation, that persist for minutes to hours, is mediated by the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) and/or phospholipase A_2 (PLA_2). 1,25-$(OH)_2D_3$ has been shown to increase mRNA and protein levels of some PLC isoforms (βI, βII, γI and δI) (Pillai et al., 1995, Xie and Bikle, 1997), to regulate PLC activity in skeletal muscle cells (Morelli et al., 1996), to regulate the activity of PLA_2 in growth zone chondrocytes and in osteoblasts-like cells (Sylvia et al., 1998, Schwartz et al., 1992), and to regulate PLD activity (Caco-2 and skeletal muscle cells) and expression (keratinocytes) (Khare et al., 1999, Morelli et al., 1996, Griner et al., 1999). In mouse epidermal keratinocytes treatment with 1,25-$(OH)_2D_3$ induced PLD-1 (a PLD isozyme) expression that was significant at 8 hours and peaked at 24 hours (Griner et al., 1999). Moreover, this effect was accompanied by an increase in total PLD-1 activity. In another study, 1,25-$(OH)_2D_3$ was reported to induced an increase in DAG levels in confluent mouse osteoblasts (Grosse et al., 1993). Moreover, Morelli et al. reported that 1,25-

$(OH)_2D_3$ induced a rapid (15 seconds) release of inositol 1,4,5-trisphosphate (IP_3) and DAG in myoblasts that correlated with translocation of PKC from the cytosol to the cell membrane (Morelli et al., 1993). Therefore, 1,25-$(OH)_2D_3$ may induce PKCα translocation by regulating the activity of phospholipases and inducing metabolism of phosphoinositols. For instance, regulation of phospholipids metabolism by 1,25-$(OH)_2D_3$ has been reported in rat hepatocytes (Baran et al., 1989), chick myoblasts (de Boland et al., 1994), confluent mouse osteoblasts (Grosse et al., 1993), and in the nuclear envelope of ROS 17/2.8 cells (Sorensen and Baran, 1995).

Another possibility may be that 1,25-$(OH)_2D_3$ directly binds and activates PKCα. Vitamin A metabolites have been shown to induce PKC translocation in several cell lines including, mouse melanoma cells (Gruber et al., 1995), human leukemia cells (Zauli et al., 1996), and rat splenocytes (Zorn and Suaro, 1995). Very recently, Hämmerling's laboratory reported that retinoids serve as ligands and coactivators of PKC (Hoyos et al., 2000, Iham et al., 2000). The cystein-rich domains of PKCα, δ and μ were tested as GST fusion proteins for their capacity to bind several retinoids (retinol, 14-hydroxy-retro-retinol, anhydroretinol and retinoic acid). All the retinoids tested bound to the cyteine-rich motif of the PKCs in vitro in nanomolar concentrations. Moreover, retinol and 14-hydroxy-retro-retinol, in the presence of oxygen species, significantly enhanced the activation of PKC. They hypothesize that hydroxylated retinols by binding to the zinc-finger domains allow the oxidation of critical cysteine residues. This cause the zinc finger to open and by a conformational change the PKC molecule unfolds and translocates to the cell membrane. Therefore, binding of 1,25-$(OH)_2D_3$ to PKCα may not be ruled out.

Finally, another possibility may be that 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by regulating its gene expression. However, our results indicated differently. Regulation of PKCα, PKCβI and PKCβII gene expression by 1,25-$(OH)_2D_3$ in HL-60 cells was reported (Obeid et al., 1990, Solomon et al., 1991). On the other hand, we determined that 1,25-$(OH)_2D_3$ did not significantly alter PKCα mRNA levels in ROS 17/2.8 cells. Therefore, 1,25-$(OH)_2D_3$ appears to increase PKCα nuclear levels in this cell line by mechanism independent of gene expression.

As discussed above, several reports have suggested that some of the effects of 1,25-$(OH)_2D_3$ may be mediated by mechanisms independent of gene regulation (reviewed also in: Norman and Collins, 1996, Revelli et al., 1998). These membrane-initiated responses (i.e. Ca^{2+} translocation and PKC activation) are postulated to be mediated by a membrane receptor (pmVDR) for vitamin D (Norman and Collins, 1996, Nemere and Farach-Carson, 1998, Nemere and Campbell, 2000). It is important to mention that 1,25-$(OH)_2D_3$ did not alter PKCα association with the plasma membrane, but induced translocation of PKCα to the nuclei. Nevertheless, it appears that in ROS 17/2.8 cells the 1,25-$(OH)_2D_3$-mediated regulation of PKCα is independent of transcriptional regulation. However, the signaling mechanism by which 1,25-$(OH)_2D_3$ induces translocation of PKCα to the nuclei in this cell line remains to be determined. Figure 1 summarizes the possible mechanisms discussed in this section.

PKCα role in 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin gene expression

Many of the physiological functions mediated by PKC involve nuclear events, including regulation of gene expression. For instance, PKC is known to phosphorylate RNA Polymerase II (*in vitro*), Lamin B (*in vitro*/*in vivo*), DNA polymerase α and β (*in vitro*) and several histones (*in*

vitro) (reviewed in: Buchner, 1995). PKC has also been implicated in the transcriptional activation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes (Auwerx et al., 1989), down-regulation of the β1-adrenergic receptor gene (Li et al., 1998), and the posttranscriptional regulation of the H-ferritin, GAP-43 and ribonucleotide reductase R1 genes (Pang et al., 1996, Perrone-Bizzozero et al., 1993, Chen et al., 1994).

Previous work suggested PKC to be involved in the actions of 1,25-$(OH)_2D_3$ in bone with respect to osteocalcin regulation (van Leeuwen et al., 1992, Desai et al., 1995, Mosavin, 1996). In chapters 2 and 3 we reported that 1,25-$(OH)_2D_3$ specifically increased PKCα levels in the nuclei in ROS 17/2.8 cells, suggesting a role for this isoform in 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin synthesis. As discussed in chapter 4, PKC activators and inhibitors were used in the past to study the involvement of PKC in the regulation of osteocalcin expression. However, the data is fraught with discrepancies such as conflicting results between reports (discussed in detail in Chapter IV). To study the role of PKCα in the regulation of osteocalcin expression we took a different approach. We overexpressed PKCα in ROS 17/2.8 cells, and determined the effect of PKCα overexpression on steady state and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA levels. We determined that overexpression of PKCα inhibited by over 75% the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA levels, without affecting basal osteocalcin levels. Several possible mechanisms by which PKCα may regulate osteocalcin gene expression are discussed therein.

First, our laboratory has demonstrated that 1,25-$(OH)_2D_3$ regulates osteocalcin expression mainly at the posttranscriptional level (Mosavin and Mellon, 1996). A stem-loop structure in the 5'-

UTR mRNA has been postulated to play a role in this 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA (Mosavin, 1996). Moreover, several cytoplasmic factors were shown to bind to this region. The affinity of these factors for the stem-loop structure remained the same in the present and absence of 1,25-$(OH)_2D_3$ as determined by RNA band shift assays (Mosavin, 1996). Therefore, there exists the possibility that these factors are activated and/or inactivated by phosphorylation/dephosphorylation events. For instance, phosphorylation of these factors by PKCα may alter their association with the 5'UTR. This may induce degradation of the osteocalcin message. Therefore, by phosphorylating these factors PKCα may block the 1,25-$(OH)_2D_3$-mediated stabilization of osteocalcin mRNA.

Second, another possibility is that PKCα inhibits 1,25-$(OH)_2D_3$-induced osteocalcin levels by regulating vitamin D receptor (VDR) expression and activity. Krishnan and Feldman reported that activation of PKC by PMA resulted in down-regulation of VDR abundance in a time- and dose-dependent manner (Krishnan and Feldman, 1991). Moreover, both oleoyl acetyl glycerol (a DAG analog) and A23187 (a Ca^{2+} ionophore) also suppressed VDR abundance. This effect appeared to be mediated at the level of gene expression as determined by northern blotting. On the other hand, phosphorylation of the VDR by PKC has been reported (Hsieh et al., 1993, Hsieh et al., 1991). In these reports VDR was a substrate for PKCβ both *in vitro* and *in vivo*. Ser51 appears to be the site of PKCβ-mediated phosphorylation. Mutation of Ser51 markedly inhibited transcriptional activity by 1,25-$(OH)_2D_3$, suggesting that Ser51 could play a significant role in 1,25-$(OH)_2D_3$-dependent transcriptional activation. However, Brown and Deluca demonstrated that phosphorylation of VDR occurs in a 23-KDa segment of the carboxy terminal (Brown and DeLuca, 1991). Hsieh's results are in contradiction with Brown's report since Ser51 is localized

outside the 23-KDa fragment. Nevertheless, regulation of VDR expression and/or activity by PKCα may not be ruled out.

Third, the vitamin D receptor forms a heterodimeric complex with the retinoic acid receptor (RXR). This complex is necessary for optimal induction of gene expression. McDonald and coworkers reported that 9-*cis* retinoic acid (a RXR ligand) inhibited 1,25-$(OH)_2D_3$-activated expression of osteocalcin gene (MacDonald et al., 1993). Their hypothesis is that 9-*cis* retinoic acid inhibits 1,2-$(OH)_2D_3$ responsiveness by diverting RXR toward RXR-dependent transcriptional signaling. PKCα may regulate RXR activity and prevent VDR-RXR heterodimeric formation. Phosphorylation of retinoic acid receptor α (RARα) by PKC has been reported (Delmotte et al., 1999). However, to our knowledge, PKC-mediated phosphorylation of RXR has not been reported.

PKCα may modulate the activity of transcription factors involved in the regulation of osteocalcin gene expression. For instance, the osteocalcin promoter contains three AP-1 sites. One of these sites lies in the vitamin D response element (VDRE). Aslam et al. demonstrated that this AP-1 element is necessary to support responsiveness to vitamin D (Aslam et al., 1999). However, it has been shown that binding of transcription factors (i.e. c-fos/c-jun heterodimer) to this AP-1 site block the binding of the VDR/RXR complex to the VDRE (Owen et al., 1990). PKCα may phosphorylate and therefore induce transcription factors to bind to this AP-1 site, blocking the access of the VDR/RXR complex to the VDRE.

On the other hand, as discussed in chapter 4, there exists the possibility that other PKC isoforms may be involved in the 1,25-$(OH)_2D_3$-mediated regulation of osteocalcin expression. First, we didn't study PKCβII and ι regulation by 1,25-$(OH)_2D_3$ and these isoforms are known to be expressed in ROS 17/2.8 cells (Sanders and Stern, 1996). Second, opposing effects by PKC isozymes has been reported. Mochly-Rosen's laboratory reported that inhibition of PKCε activity inhibited, whereas inhibition of PKCδ enhanced, hormone- and PMA-induced contraction rate in cardiac myocytes (Johnson et al., 1996, Csukai and Mochly-Rosen, 1998). In another report, Mischak and coworkers determined that while overexpression of PKCδ in NIH 3T3 cells induced significant changes in morphology and caused the cells to growth more slowly, overexpression of PKCε resulted in increased growth rates and no change in cell morphology (Mischak et al., 1993). These results taken together suggest that more than one isoform may be implicated in PKC-mediated regulation of a particular cellular function.

Finally, even though we determined that 1,25-$(OH)_2D_3$ induces PKCα nuclear translocation, there exist the possibility that PKCα regulates osteocalcin gene expression by a mechanism independent of nuclear translocation. PKCα may phosphorylate another kinase present in the cytosol and phosphorylation of this kinase triggers a nuclear-directed kinase cascade.

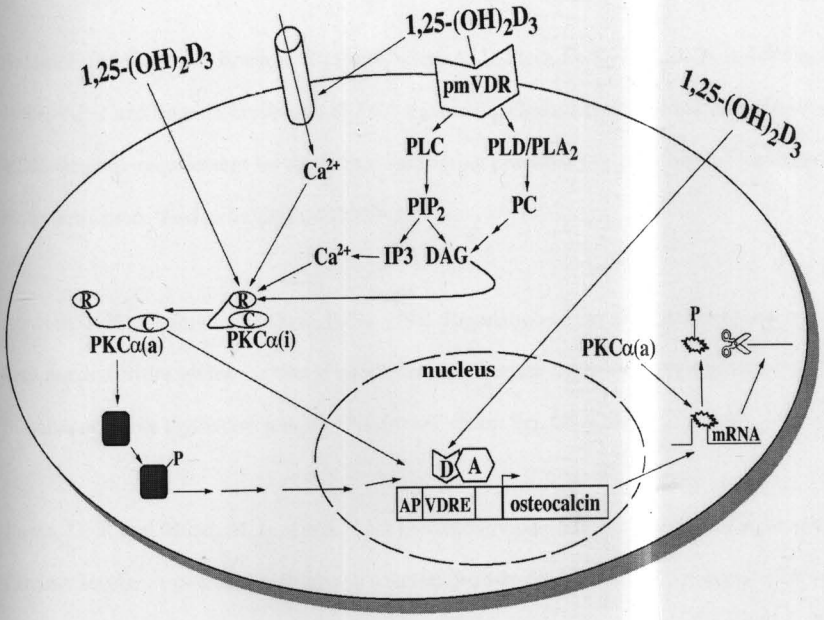
We have presented evidence that 1,25-$(OH)_2D_3$ regulates PKCα subcellular localization in ROS 17/2.8 cells. We proposed that 1,25-$(OH)_2D_3$ induces translocation of PKCα from the cytosol to the nuclei. Once in the nuclei and by unknown mechanism(s), PKCα blocks the 1,25-$(OH)_2D_3$-mediated increase in osteocalcin mRNA levels. To our knowledge, this is the first time regulation of PKC expression and activity is reported in a bone cell line. Moreover, this is the

first report that postulates a role of a particular PKC isoform in the regulation of osteocalcin gene expression.

Figure 1. Schematic representation of possible signaling mechanisms involved in PKCα regulation by 1,25-$(OH)_2D_3$ and in the PKCα-mediated regulation of osteocalcin gene expression. The illustration represents data obtained from our studies in ROS 17/2.8 cells and from reports obtained in different cell types. 1,25-$(OH)_2D_3$ may increase $[Ca^{2+}]_i$ levels by two mechanisms: by modulating dihydropyridine-sensitive Ca^{2+} currents and by receptor-mediated (pmVDR) activation of phosphoinosites metabolism. Once intracellular Ca^{2+} levels are elevated, binding of Ca^{2+} to PKCα's regulatory domain induces PKCα translocation. Generation of diacylglycerol (DAG) may be another mechanism by which 1,25-$(OH)_2D_3$ regulates PKCα subcellular distribution. Binding of 1,25-$(OH)_2D_3$ to a vitamin D receptor located in the plasma membrane (pmVDR) induces hydrolysis of either phosphatidylinositol 4,5-bisphosphonate (PIP_2) by phospholipase C (PLC) and/or phosphatidylcholine (PC) by phospholipase D (PLD) and phospholipase A_2 (PLA_2), resulting in generation of DAG. Finally, 1,25-$(OH)_2D_3$ may directly bind to PKCα's regulatory domain, inducing a conformational change that unfold the PKCα molecule, resulting in translocation to the nucleus. How 1,25-$(OH)_2D_3$ induces translocation of PKCα to the nucleus and not the plasma membrane in ROS 17/2.8 cells is unknown.

Once activated, PKCα translocates to the nuclei were by unknown mechanism(s) it regulates osteocalcin gene expression. First, PKCα may regulate osteocalcin expression by modulating vitamin D receptor (VDR) activity. Phosphorylation of this nuclear receptor by PKCα may alter the formation of the vitamin D receptor/retinoic acid receptor (VDR/RXR) complex. This would result in inhibition of VDR/RXR binding to vitamin D response elements (VDRE) present in the osteocalcin promoter with the concomitant inhibition of osteocalcin mRNA production. VDR

and RXR are denoted in the illustration as D and A, respectively. On the other hand, PKCα may also regulate RXR activity and prevent formation of the VDR/RXR complex. PKCα may modulate the activity of transcription factors involved in the regulation of osteocalcin gene expression (not shown). Phosphorylation of these transcription factors may induce their binding to AP-1 sites (AP), blocking VDR/RXR access to the osteocalcin promoter. On the contrary, PKCα may regulate osteocalcin gene expression by a mechanism independent of nuclear translocation. For example, PKCα may phosphorylate another kinase (represented as a black square) present in the cytosol and phosphorylation of this kinase triggers a nuclear-directed kinase cascade. Finally, PKCα may inhibit 1,25-$(OH)_2D_3$-activated osteocalcin mRNA levels at the posttranscriptional level. PKCα may phosphorylate factors that bind and protect the 5'-UTR on osteocalcin mRNA of degradation. Phosphorylation of these cytoplasmic factors (only one factor is depicted bound to the 5'-UTR) may result in their inactivation. These mechanisms are explained in more detail in the test.


1,25-(OH)2D3
1,25-(OH)2D3
pmVDR
1,25-(OH)2D3
PLC
PLD/PLA2
Ca2+
PIP2
PC
Ca2+
IP3
DAG
R
C
PKCα(a)
R
C
PKCα(i)
nucleus
P
PKCα(a)
mRNA
P
D
A
AP
VDRE
osteocalcin

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Appendix

Regulation of protein kinase Cα gene expression by 1,25-dihydroxyvitamin D_3 in rat osteoblast cells (ROS 17/2.8)

(research done in collaboration with Sue Stein and Hak Auth)

ABSTRACT

We determined PKCα gene regulation by 1,25-$(OH)_2D_3$ since previously we reported that 1,25-$(OH)_2D_3$ increased PKCα protein levels in the nuclei in ROS 17/2.8 cells. Treatment of ROS 17/2.8 cells with 20 nM 1,25-$(OH)_2D_3$ increased PKCα mRNA levels at 30 minutes, but not at 1 hour, 6 hours, 12 hours and 24 hours, as determined by RT-PCR. To further determine if translocation if the main mechanism by which 1,25-$(OH)_2D_3$ increased PKCα nuclear levels, we evaluated nuclear fractions to determine if the 1,25-$(OH)_2D_3$-mediated increase in PKCα protein levels would be affected in the presence of transcriptional inhibition. Inhibition of transcription with actinomycin D did not alter the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels. Taken together this data indicates that the induction of PKCα nuclear levels by 1,25-$(OH)_2D_3$ occurs mainly by translocation and is independent of gene expression.

INTRODUCTION

It is well established that 1,25-$(OH)_2D_3$ controls mineral homeostasis by signal transduction mechanisms involving an intracellular receptor with the modulation of gene expression (Darwish and DeLuca, 1993). Vitamin D receptor expression has been reported in over 35 tissues and more than 50 genes are known to be regulated by 1,25-$(OH)_2D_3$ (Christakos, 1996, Norman and Collins, 1996). However, several reports suggest that some of 1,25-$(OH)_2D_3$ biological effects may be independent of gene regulation (reviewed in: Norman and Collins, 1996, Revelli et al., 1998). These so called "non-genomic" effects include: 'transcaltachia" (rapid Ca^{2+} translocation) in chick enterocytes (Nemere et al., 1984, Nemere and Norman, 1987), increases

in $[Ca^{2+}]_i$, in nuclear Ca^{2+} and phospholipids metabolism in rat hepatocytes (Baran and Milne, 1986, Baran et al., 1989), induction of phospholipids hydrolysis and regulation of phospholipases activity in chick myoblasts (de Boland et al., 1994, Morelli et al., 1996), and increases in $[Ca^{2+}]_i$ in chick skeletal muscle cells (Capiati et al., 2000),

"Non-genomic" effects of 1,25-$(OH)_2D_3$ have also been reported in osteoblasts. 1,25-$(OH)_2D_3$ has been shown to increase $[Ca^{2+}]_i$ in confluent mouse osteoblasts, in ROS 17/2.8 and ROS 24/1 cells (Lieberherr, 1987, Civitelli et al., 1990, Baran et al., 1991), to modulate dihydropyridine-sensitive Ca^{2+} currents in ROS 17/2.8 cells (Caffrey and Farach-Carson, 1989), and to alter phospholipids metabolism in confluent mouse osteoblast and in ROS 17/2.8 cells (Grosse et al., 1993, Sorensen and Baran, 1995). In addition, 1,25-$(OH)_2D_3$ has been shown to regulate PKC activity in several cell types (Simboli-Campbell et al., 1994, Berry et al., 1996, Bissonnette et al., 1994, Capiati et al., 1999, Simpson et al., 1998).

Previously, we reported that 1,25-$(OH)_2D_3$ increased PKCα levels in the nuclei in ROS 17/2.8 cells. This increase was accompanied by a concomitant decrease in cytosolic PKCα levels, suggesting translocation. However, this 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels may result from an increase in PKCα gene expression since 1,25-$(OH)_2D_3$ is known to regulate gene expression. For instance, 1,25-$(OH)_2D_3$ was reported to increase PKCα (and PKCβs) steady state mRNA levels in HL-60 cells (Obeid et al., 1990, Solomon et al., 1991). Nevertheless, 1,25-$(OH)_2D_3$ has also been shown to induce translocation of PKCα in several cells, including kidney (Simboli-Campbell et al., 1994), leukemia (Berry et al., 1996), colon cancer (Bissonnette et al., 1994), and skeletal muscle (Capiati et al., 2000) cells.

The experiments outlined in this section were designed to determined if gene expression and/or translocation is(are) involved in the 1,25-$(OH)_2D_3$-mediated up regulation of nuclear PKCα levels. First, we studied regulation of PKCα gene expression by 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells by RT-PCR. Then we determined the effect of transcriptional inhibition in the 1,25-$(OH)_2D_3$-mediated increase in PKCα levels in the nuclei. Here we report that 1,25-$(OH)_2D_3$ did not significantly alter PKCα mRNA levels. Moreover, inhibition of transcription by actinomycin D did not affect the induction of PKCα nuclear levels by 1,25-$(OH)_2D_3$. This data strongly suggests that 1,25-$(OH)_2D_3$ increases PKCα nuclear levels mainly by translocation and gene expression does not appear to play a significant role.

MATERIALS AND METHODS

Materials 1,25-$(OH)_2D_3$ was kindly provided by Dr. Hector F. DeLuca (University of Wisconsin-Madison). Rabbit polyclonal IgG antibody that recognizes PKCα and peroxidase-conjugated anti-rabbit IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, California). cPKCα antibody was raised against a peptide mapping at the carboxy terminus of cPKCα of human origin. cPKCα antibody recognizes PKCα of mouse, rat and human origin. Actinomycin D was purchased from Sigma (St. Louis, MO). The PKCα primers were purchased from Integrated DNA Technologies (Coralville, IA). Reverse Transcription System, PCR Core System I and PCR markers were purchased for Promega (Madison, WI). QuantumRNA 18S Internal Standards and DnaseI were purchased form Ambion, Inc. (Austin, TX).

Cell culture ROS 17/2.8 cells were maintained in Ham's F-12 (1:5) culture medium supplemented with 5% (vol/vol) defined fetal bovine serum (Hyclone, Utah), penicillin (100 u/ml), and streptomycin (0.1 mg/ml). Cells were propagated continuously at 37^0C under a humidified 5% CO_2: 95% air mixture. For the experiments, cells were plated at a density of 1.0 x 10^4 cells/cm^2 on 55 cm^2 petri dishes and allowed to adhere overnight. When the cells reached 50-70% confluence they were treated with either ethanol (as control), 1,25-$(OH)_2D_3$ (20 nM) or PMA (100 nM) for a specified amount of time. Experiments were carried out on cells between the 5th and 30th passage. For the actinomycin D (AD) experiments, cells were treated with 5 μg/ml of AD one hour prior to sample treatments.

RNA isolation Total RNA was isolated using the guanidinium-pheno-bromochloropropane method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987, Chomczynski and Sacchi, 1995). Briefly, approximately 2 x 10^6 cells were suspended in 200 µl of homogenization buffer (4 M guanidinium thiocynate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol). Addition of 20 µl of 2 M sodium acetate, 200 µl water-saturated phenol, and 40 µl bromochloropropane followed. The tubes were vortexed for 10 seconds and placed on ice for 15 minutes. The tubes were centrifuged at 10,000 x g for 20 minutes at 4^0C and the upper phase, containing the RNA, was transferred to another tube. RNA was precipitated by incubating with isopropanol (1:1) overnight at -20^0C. The RNA was pelleted by centrifugation at 10,000 x g for 20 minutes at 4^0C. The pellet was redissolved in 50 µl homogenization buffer, and 50 µl of isopropanol was added to the tubes. The RNA was reprecipitated overnight at -20^0C. The RNA was pelleted by centrifugation at 10,000 x g for 15 minutes at 4^0C, washed with 70% ethanol and resuspended with diethyl pyrocarbonate (DEPC)-treated water.

Reverse Transcription (RT) Reverse transcription was performed at 42^0C for 50 minutes using 1 µg of RNA and 15 U/µg of RNA of AMV Reverse Transcriptase. Random Primers (0.5 µg/µg of RNA hexamer oligonucleotides) were used to prime the transcription reaction. The reaction mixture (20 µl) contained the following: 1X buffer [10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Triton X-100], 5 mM $MgCl_2$, 1 mM each dNTP, and 1 U/µl Recombinant RNasin© Ribonuclease Inhibitor. The reaction was initially incubated for 10 minutes at 25^0C to maximize the Random Primers activity. After completion of the 50 minutes transcript incubation, the

reaction was heated at 99^{0}C for 5 minutes to inactivate the AMV Reverse Transcriptase. The obtained cDNA product was stored at -20^{0}C.

Polymerase Chain Reaction (PCR) Promega's PCR Core System I was used for PCR amplification. One set of specific PKCα primers was used in the reaction. The gene sequence chosen for PKCα-5' and PKCα-3' primers represented an area of the V3 region (variable region #3) of PKCα (Ishizuka et al., 1996). The PKCα-5' sequence used was ACCCTCAGTGGAATGAGTCCTTCACGT. The PKCα-3' sequence chosen was TTAGATGGCTGCTTCCTGTCTTCTGAA. The pair of primers produced cDNA fragment 328 base pairs long. The reaction volume for the PCR amplification was 50 µl. A master mix was prepared to maximize precision. The mix consisted of 1.5 mM $MgCl_2$, 1.25 units Taq DNA Polymerase and 200 µM each base in a Nucleotide Mix. In addition, 1 µg cDNA and 25 pmole of the 3' and 5' PKCα specific primers were added to each tube. To correct for variation in amplification, Ambion's QuantumRNA 18S Internal Standard was employed. The manufactured recommended a primer-to-competimer ratio of 1:9 for extremely rare, 2:8 for rare and 3:7 for moderately expressed transcripts. After densitometric analysis, an optimal primer-to-competimer ratio of 2:8 proved most effective for PKCα quantitation. PCR competimer quantitation reactions incorporated 4 pmole of the primer and 16 pmole competimer mixture, in addition to the above reactants, in each tube. The samples were overlaid with light mineral oil and centrifuged briefly before coamplification. PCR was performed at the following parameters: denaturation at 95^{0}C for 30 seconds, annealing at 55^{0}C for 1 minute followed by extension soak at 72^{0}C for 2 minutes. A Perkin Elmer DNA Thermal Cycler 480 (Norwalk, CT) was utilized for PCR. Initially, PCR amplification of non-reverse transcription mRNA was completed for each

sample of mRNA to demonstrate purity of RNA and lack of genomic DNA before RT. The linear range of amplification was determined for each cDNA sample analyzed in an effort to maintain the exponential rate of production (Larrick and Siebert, 1995). Cycles ranging from 12 to 42 were examined. Consistently, calculations showed 26 cycles amplification fell within the linear range and, therefore, 26 cycles were utilized for quantitation. A 2% agarose gel stained with ethidium bromide was used to visualize the PCR products.

Preparation of nuclear fraction Nuclear fractions were prepared as described before with some modifications (Delude et al., 1994). All the steps were performed at 4^0C. Cells were washed and collected in a chilled 1X PBS buffer solution. The cell suspension was centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 600 µl of Buffer A [10 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.3 M sucrose, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. Nonidet P-40 was added to 0.6% (vol/vol) and the tubes were vortexed at setting 10 with a Vortex mixer (Spectra-Physics, California) for 20 seconds. Nuclei were harvested by centrifugation of the samples at 7,200 x g for 30 seconds. The pellet (nuclei) was resuspended in 300 µl of Buffer B [20 mM Tris (pH 7.8), 5 mM $MgCl_2$, 320 mM KCl, 0.5 mM DTT, 1% protease inhibitor cocktail solution], and the tubes were placed on ice for 15 minutes. The samples were centrifuged at 13,500 x g for 15 minutes. The resulting supernatant (nuclear extract) was collected.

Immunoblotting For immunoblotting, samples (nuclear fractions) were diluted 1:1 (vol/vol) in 2X sample buffer [12 mM Tris, 1.5 mM EDTA, 20 mM DTT, 2% SDS, 20% glycerol, and 0.2%

bromophenol blue] and solubilized by boiling for 5 minutes. The samples were electrophoresed in a 12.5% SDS-polyacrylamide minigel for 90 minutes at 160 V in 1X running buffer (250 mM Tris, 10 M glycine, and 0.01% SDS). The proteins were transferred electrophoretically to a PVDF membrane at 0.60 A (constant voltage) for 2 hours at 4^0C in 1X transblot buffer (250 mM Tris, 10 M glycine). The blots were blocked with 2.5% non-fat dry milk in TBST (1 mM Tris, 15 mM NaCl and 0.05% Tween) overnight at 4^0C. Following blocking, the blots were probed with polyclonal antibody for PKCα for 1 hour at 37^0C. The blots were washed three times for 5 minutes at 37^0C in TBST and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase for 50 minutes at 37^0C. The blots were washed subsequently 2 times for 15 minutes at 37^0C in TBST.

^{3}H-uridine incorporation measurement To establish the minimum concentration of actinomycin D (AD) necessary for maximal transcriptional inhibition, incorporation of ^{3}H-uridine in the presence and absence of AD was measured by trichloroacetic acid (TCA) precipitation as described by Mosavin (Mosavin, 1996) with some modifications. Briefly, ROS 17/2.8 cells were dosed with various concentrations of AD (2.5, 5.0 and 10 μg/ml) for 1 hour. Cells were then dosed with 10 μl (10 μCi) of ^{3}H-uridine for 1 hour. At the end of the incubation period, media was removed and cells were washed three times with 1X PBS. Cells were collected in an ice-cold 1X PBS solution and samples (in triplicate) were filtered through Whatman GF/C glass-fiber (Sigma, St Louis, MO) which were previously placed into a vacuum-driven filtration manifold. Samples were incubated with 10 ml ice-cold 5% TCA/20 mM sodium pyrophosphate for 2 minutes to precipitate nucleic acids. Filters were washed with 10 ml ice-cold 5% TCA/20 mM sodium pyrophosphate three times followed by 5 ml 70% ethanol and

allowed to dry at room temperature. Radioactivity was measured by a Packard Tri-Carb liquid scintillation analyzer (Meriden, CT). The non-specific incorporation of ^{3}H-uridine was measured by assaying the samples immediately following the addition of ^{3}H-uridine.

Detection and data analysis For RT-PCR: A 2% agarose gel stained with ethidium bromide was used to visualize the PCR products. Bands (PKCα and 18S) were quantified by using the Molecular Dynamics 300 A Computing Densitometer (Sunnyvale, California). PKCα levels were corrected to the 18S ribosomal band and expressed relative to control. Statistical comparison were made by using *t*-test (Statistica, StatSoft) for independent sample means at 95% confidence level. The data is expressed as the mean ± the standard error of the mean.

PKCα expression on western blots was detected using the LumiGlo chemiluminescent substrate kit (Kirkegaard & Perry Laboratories, Maryland) and blots were exposed to x-ray film (RX-U, FujiFilm) with Quanta III intensifying screens. Bands were quantified by using the Molecular Dynamics 300 A Computing Densitometer (Sunnyvale, California). The range of the linearity of PKCα density measurements was calculated to be between 5 μg to 50 μg of total protein. To decrease variability in PKCα density measurements between experiments the amount of total protein electrophoresed and the exposure time of blots to x-ray films were kept constant. PKCα levels were expressed relative to control. Statistical comparisons were made by 2 ways analysis of variance (ANOVA) (Statistica, StatSoft). The data is expressed as the mean ± the standard error of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

Parameters for the RT-PCR analysis

We determined regulation of PKCα gene expression by 1,25-$(OH)_2D_3$ by RT-PCR since northern blot analysis failed to produce a detectable signal for PKCα mRNA. For northern analysis GAPDH, a housekeeping gene, was used as an internal control. A GAPDH signal was obtained in the northern analysis, indicating that the protocol was done correctly. This also indicates that the levels of PKCα mRNA were too low to be detected by northern blotting.

The parameters used for the RT-PCR analysis are described in the materials and methods section and as determined by Stein (Stein, 1999). First, to compensate for variability and to provide accurate quantitation of the PCR products, 18S rRNA was used as an internal standard. rRNA has been shown to display less variability compared to other housekeeping genes (i.e. β-actin) (deLeeuw et al., 1989). After densitometric analysis, an optimal primer-to-competimer ratio of 2:8 proved most effective for PCR quantitation. Second, the quantity of template cDNA to be used for the PCR reaction was determined. Several concentrations of RNA were used as the starting material for RT and 1 μg of RNA was shown to produce sufficient PKCα cDNA for the PCR analysis.

For the PCR analysis, the appropriate number of cycle amplification was determined. Each amplification cycle of PCR will increase DNA production in a parallel relationship up to a certain point (Saiki et al., 1985), were production will plateau. The most precise PCR analysis of a gene is possible in the liner range of the amplification reaction. To determine the optimal

number of amplification cycles 12 to 42 cycles were run and the product from each cycle was visualized on 2% agarose gels stained with ethidium bromide. Cycles 26-30 were in the linear range of amplification. Twenty-six cycles was selected since it was at the beginning of the linear range. Finally, we determined that the PCR product was indeed PKCα by restriction enzyme analysis.

Effect of 20 nM 1,25-$(OH)_2D_3$ on PKCα mRNA levels

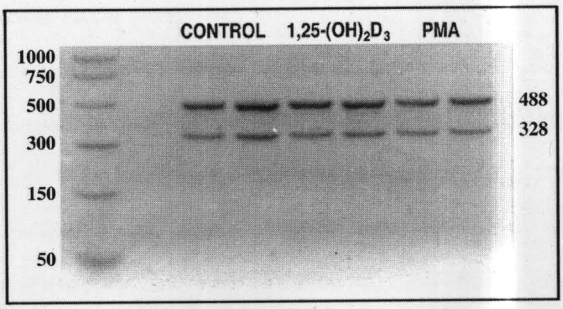
ROS 17/2.8 cells were treated with either ethanol (as vehicle), 20 nM 1,25-$(OH)_2D_3$ or 100 nM PMA for 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours. PMA was used as a positive control. PMA is known to activate conventional (α, βI, βII and γ) and novel [δ, ε, η(L) and μ] PKCs (Nishizuka, 1995). However, regulation of PKC gene expression by PMA has not been reported. Therefore, PMA treatment was not expected to alter PKCα mRNA levels. After treatment, RNA was isolated and RT-PCR analysis was performed.

Figure 1A represents a gel for expression PKCα mRNA levels in ROS 17/2.8 cells after 12 hours treatment. Figure 1B summarizes the effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα mRNA levels at different times of treatment. As shown in the figure, treatment with 1,25-$(OH)_2D_3$ for 30 minutes increased PKCα levels by 1.4-fold. This increase in PKCα mRNA levels was low but statistically significant as assessed by student t-test ($p < 0.05$). However, no significant change in PKCα mRNA levels was observed with 1,25-$(OH)_2D_3$ treatment at any of the other time points examined. As expected, no significant change in PKCα mRNA levels was observed with PMA treatment at any time.

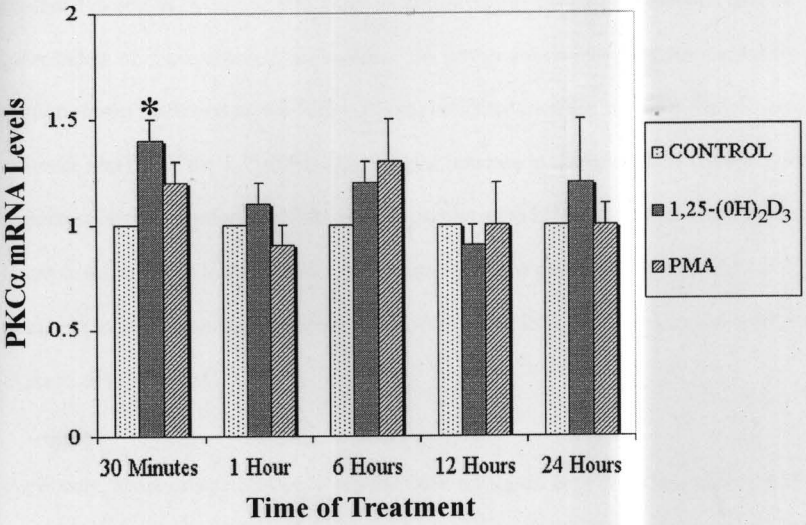
Figure 1. Effect of 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA on PKCα mRNA levels

Panel A: Example of ethidium bromide stained gel showing PKCα mRNA levels in ROS 17/2.8 cells at 12 hours treatment with ethanol (as vehicle), 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA. RNA was isolated and RT-PCR was performed. PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide. The 488 band represents the 18S ribosomal RNA and 328 band represents PKCα. **Panel B**: Densitometric analysis of PKCα mRNA levels in ROS 17/2.8 cells treated with ethanol (as vehicle), 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA for 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours. PKCα was corrected to the 18S ribosomal band and expressed relative to control. The statistical significance was assessed by student t-test for independent sample means. The error bars indicate the standard error of the mean of at least three independent experiment performed in duplicates. ***$p < 0.05$ vs. control**

A.


CONTROL
1,25-(OH)2D3
PMA
1000
750
500
300
150
50
488
328

B.


PKCα mRNA Levels
2
1.5
1
0.5
0
*
30 Minutes
1 Hour
6 Hours
12 Hours
24 Hours
Time of Treatment
CONTROL
1,25-(0H)2D3
PMA

Inhibition of transcription with actinomycin D did not alter the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels

The data presented in chapters 2 and 3 demonstrated that 1,25-$(OH)_2D_3$ increased PKCα levels in the nuclear fraction. This increase was accompanied by a decrease in PKCα levels in the cytosol (as detected by immunofluorescence), suggesting translocation. However, 1,25-$(OH)_2D_3$ is known to effect its actions by interacting with an intracellular receptor with the concomitant regulation of gene expression. As demonstrated above, 1,25-$(OH)_2D_3$ did not significantly alter PKCα mRNA levels in ROS 17/2.8 cells. This strongly suggests that the 1,25-$(OH)_2D_3$-mediated increased in nuclear PKCα levels occurs mainly by translocation and may be independent of transcriptional mechanisms. To further substantiate this, we studied the effect of transcriptional inhibition on the 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels. The rationale was that if the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα remains unchanged regardless of transcriptional inhibition, then translocation is likely the event that increases PKCα levels in the nuclei. On the contrary, a decrease in PKCα nuclear levels after 1,25-$(OH)_2D_3$ treatment in the presence of actinomycin D (AD) would indicate that regulation of PKCα gene expression in involved.

Previously, Mosavin and Mellon determined that a 5 μg/ml dose of AD proved to be effective in blocking transcription (95% inhibition) as determined by ^{3}H-uridine incorporation, and low toxicity (90% cell viability up to twelve hours of treatment), (Mosavin and Mellon, 1996). To verify those experiments, incorporation of ^{3}H-uridine in the presence and absence of AD was measured by trichloroacetic acid (TCA) precipitation as described under materials and methods. Figure 2 summarized the effect of AD (2.5, 5.0 and 10 μg/ml) on ^{3}H-uridine incorporation. As

shown in the figure, all three doses inhibited ^{3}H-uridine incorporation by over 90%. 5 μg/ml was selected at the AD dose for the experiments.

For the actinomycin D experiments, ROS 17/2.8 cells were dosed with 5 μg/ml of AD 1 hour prior to sample treatments. Cells were treated with either ethanol (as vehicle), 20 nM 1,25-$(OH)_2D_3$ or 100 nM PMA for 1 hour. After treatment, nuclear fractions were prepared and western blot analysis was performed. The same experiments were done in the absence of AD for statistical comparisons. Figure 3A represents an immunoblot for expression of PKCα levels in the nuclear fraction after 1 hour treatment in the presence of transcriptional inhibition. Figure 3B summarizes the effect of 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA in the presence and absence of AD. As shown in this figure, treatment with 1,25-$(OH)_2D_3$ for 1 hour in the absence of transcriptional inhibition increased PKCα nuclear levels by 2.4-fold compared to control (no AD). In the presence of AD, 1,25-$(OH)_2D_3$ increased PKCα nuclear levels by 2.3-fold compared to control (AD). In comparison, PMA treatment increased PKCα nuclear levels by 1.8-fold and 2.5-fold, respectively. A two ways analysis of variance was used to determine the effect of the presence of AD on nuclear PKCα levels. The analysis showed that the presence of AD did not alter the 1,25-$(OH)_2D_3$- and PMA-mediated increase in nuclear PKCα levels, indicating that the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels is independent of gene expression.

Figure 2. Inhibition of ^{3}H-uridine incorporation by actinomycin D

Incorporation of ^{3}H-uridine was determined in ROS 17/2.8 cells dosed with various concentrations (2.5, 5.0 and 10 μg/ml) of actinomycin D by trichloroacetic acid (TCA) precipitation. The percentage of ^{3}H-uridine incorporation in the presence of AD was compared to control (no AD). The error bars indicate the lower and higher values of three determinations. AD: actinomycin D

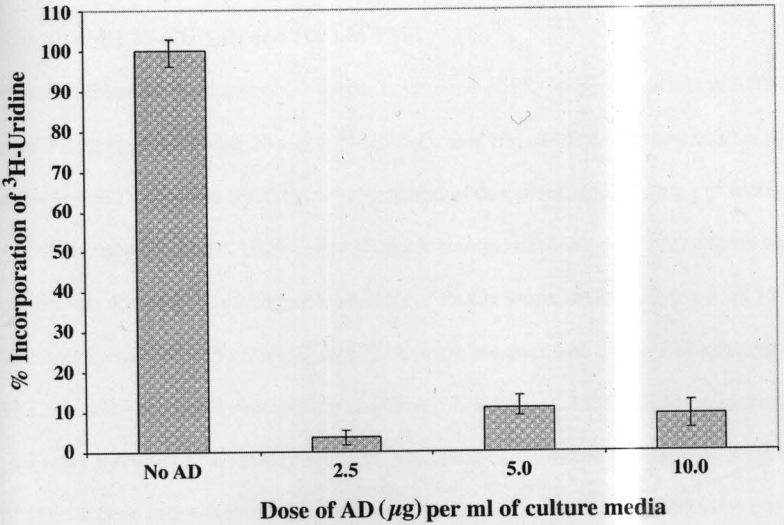

% Incorporation of ^{3}H-Uridine
110
100
90
80
70
60
50
40
30
20
10
0
No AD
2.5
5.0
10.0
Dose of AD (μg) per ml of culture media

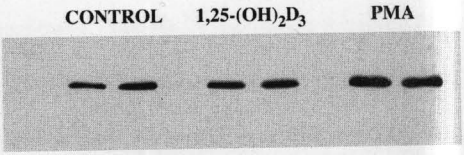
Figure 3. Effect of actinomycin D on PKCα nuclear levels in ROS 17/2.8 cells treated with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA

Panel A: Example of immunoblot for the expression of PKCα nuclear levels in ROS 17/2.8 cells after 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ and 100 nM PMA in the presence of actinomycin D. Nuclear fractions were prepared as described before. 20 μg of total protein were electrophoresed on 12.5% SDS-polyacrylamide minigels and western blot analysis was performed. **Panel B**: Densitometric analysis of PKCα levels in the nuclei in ROS 17/2.8 cells after treatment with 1,25-$(OH)_2D_3$ and PMA in the presence and absence of actinomycin D. PKCα levels were expressed relative to control. The statistical significance was assessed by a two ways analysis of variance (ANOVA). The error bars indicate the standard error of the mean of at least three independent experiments performed in duplicates. $\mathbf{p < 0.05}$ was considered statistically significance. AD: actinomycin D

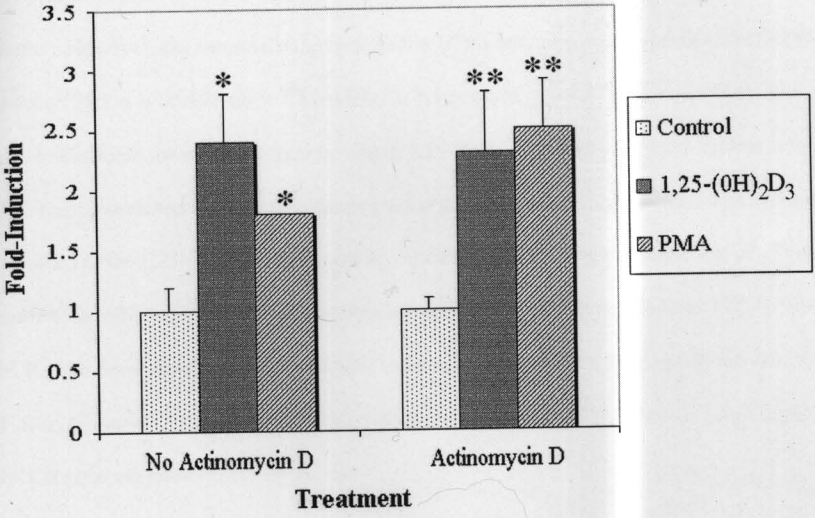
***p < 0.05 vs. control (no AD)**

****p < 0.05 vs. control (AD)**

A.


CONTROL
1,25-(OH)2D3
PMA

B.


3.5
3
2.5
2
1.5
1
0.5
0
Fold-Induction
*
*
**
**
No Actinomycin D
Actinomycin D
Treatment
Control
1,25-(0H)2D3
PMA

DISCUSSION

In chapter 2 we reported that 1,25-$(OH)_2D_3$ specifically increased PKCα levels in the nuclei in ROS 17/2.8 cells in a time dependent manner. Increases in PKCα levels were observed as early as 15 minutes upon 1,25-$(OH)_2D_3$ treatment, suggesting translocation. In chapter 3 we reported that the 1,25-$(OH)_2D_3$-mediated increase in nuclear PKCα levels was accompanied by a decrease in cytosolic PKCα levels, indicating translocation of PKCα from this compartment into the nuclei. However, the increase in nuclear PKCα levels may also indicate that 1,25-$(OH)_2D_3$ induced PKCα synthesis since 1,25-$(OH)_2D_3$ is known to regulate gene expression. Our goal was to elucidate the mechanism(s) by which 1,25-$(OH)_2D_3$ increased PKCα nuclear levels. Two different approaches were used to determined if gene expression and/or translocation is(are) involved in the 1,25-$(OH)_2D_3$-mediated up regulation of PKCα levels in the nuclei. First, we studied the effect of 1,25-$(OH)_2D_3$ treatment on PKCα mRNA levels in ROS 17/2.8 cells by RT-PCR. We determined that 1,25-$(OH)_2D_3$ increased PKCα mRNA levels at 30 minutes, but not at 1 hour, 6 hours, 12 hours and 24 hours, indicating that 1,25-$(OH)_2D_3$ does not significantly alter PKCα gene expression in this cell line.

Our studies contradict previous reports by Obeid and Solomon (Obeid et al., 1990, Solomon et al., 1991). Obeid and coworkers reported a 4-5-fold increase in PKCβ steady state mRNA levels at 24-48 hours treatment with 1,25-$(OH)_2D_3$ (Obeid et al., 1990). A similar increase was observed for PKCα mRNA levels. This 1,25-$(OH)_2D_3$-mediated increase in PKCα and PKCβ mRNA appears to occur mainly at the transcriptional level as determined by nuclear run-off

studies. Solomon and coworkers also reported increases in steady state levels of PKCα, βI and βII, with maximal induction at 48 hours after addition of 1,25-$(OH)_2D_3$ (Solomon et al., 1991). However, while Obeid and coworkers reported increases in transcriptional rates for these isoforms after treatment with 1,25-$(OH)_2D_3$, Solomon and coworkers were not able to detect changes on the PKCα, βI and βII rates of transcription. The apparent discrepancy may be due to the fact that to normalize the rate of transcription of PKCα and PKCβs genes different internal control genes were used between the studies (Solomon et al., 1991). Nevertheless, it is worth mentioning that relatively high concentrations of 1,25-$(OH)_2D_3$ were used (100 nM and 600 nM, respectively) in these studies. These concentrations are above the physiological concentrations of 1,25-$(OH)_2D_3$, and may be of no physiological relevance in respect to the regulation of PKCα and PKCβs gene expression.

The data presented above does not completely rule out the possibility that 1,25-$(OH)_2D_3$ may regulate PKCα gene expression. However, together with the data presented in chapters 2 and 3, strongly suggests that 1,25-$(OH)_2D_3$ increases PKCα nuclear levels by mechanism(s) other than gene expression. Several reports suggest that some of 1,25-$(OH)_2D_3$ biological effects may independent of gene transcriptions (reviewed in: Norman and Collins, 1996, Revelli et al., 1998). These "non-genomic" effects of 1,25-$(OH)_2D_3$ have been proposed to occur in several cell types including enterocytes (Nemere et al., 1984, Nemere and Norman, 1987), hepatocytes (Baran and Milne, 1986, Baran et al., 1989), myoblasts (de Boland et al., 1994, Morelli et al., 1996, Capiati et al., 2000) and osteoblasts (Lieberherr, 1987, Caffrey and Farach-Carson, 1989, Civitelli et al., 1990, Baran et al., 1991, Farach-Carson et al., 1991, Grosse et al., 1993, Sorensen and Baran, 1995). Moreover, 1,25-$(OH)_2D_3$ has been shown to induce translocation of PKCα to the plasma

membrane and nuclei in several cells, including kidney (Simboli-Campbell et al., 1994), leukemia (Berry et al., 1996), colon cancer (Bissonnette et al., 1994) and skeletal muscle (Capiati et al., 2000) cells.

To further substantiate that the 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels occurs by a mechanism(s) different to gene expression (i.e. translocation), our second approach was to study the effect of transcriptional inhibition on the 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels. We determined that 1 hour treatment with 20 nM 1,25-$(OH)_2D_3$ increased PKCα nuclear levels regardless of transcriptional inhibition. This in accord with previous work by Stein that showed that 30 minutes treatment with 20 nM 1,25-$(OH)_2D_3$ resulted in an increase in PKCα levels in the nuclei in the presence of transcriptional inhibition.

Finally, as expected, PMA treatment did not significantly alter PKCα mRNA levels at any time. PMA is known to activate PKC, but not to regulate its gene expression. PMA activates PKC by binding to the its regulatory domain and inducing translocation from the cytosol to other cell compartments. Therefore, we did not expect actinomycin D to affect PMA effects of PKCα nuclear levels. As expected, the PMA-mediated increase in PKCα nuclear levels was not affected by transcriptional inhibition.

In summary, the data presented in this appendix strongly suggests that the 1,25-$(OH)_2D_3$-mediated increase in PKCα nuclear levels occurs mainly by translocation and gene expression does not appear to play a significant role. Possible signaling mechanism(s) involved in the 1,25-

$(OH)_2D_3$-mediated regulation of PKCα subcellular localization are reviewed in the discussion chapter.

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