Journal of Cellular Biochemistry 94:1017–1027 (2005)

1,25-Dihydroxyvitamin D_3 Stimulated Protein Kinase C Phosphorylation of Type VI Adenylyl Cyclase Inhibits Parathyroid Hormone Signal Transduction in Rat Osteoblastic UMR 106-01 Cells

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Abstract 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) treatment of osteoblastic cells was shown previously to attenuate Parathyroid hormone (PTH) response by inhibiting adenylyl cyclase (AC) activity. In this study, we have investigated the mechanism by which $1,25(OH)_2D_3$ inhibits AC in rat osteoblastic UMR 106-01 cells. $1,25(OH)_2D_3$ treatment inhibited both PTH and forskolin-stimulated AC activity by 25%–50% within 12 min in a concentration-dependent manner suggesting a direct inhibition of the AC enzyme. Treatment with $25(OH)D_3$ had no effect on basal or stimulated AC activity. We determined the profile of AC subtypes expressed in UMR cells and found AC VI to be the dominant subtype accounting for 50% of AC mRNA. Since AC VI can be inhibited by protein kinase C (PKC) phosphorylation, we examined $1,25(OH)_2D_3$ activation of various PKC isoforms. $1,25(OH)_2D_3$ increased the membrane translocation of PKC-βI, -δ, and -ζ with a concomitant increase in PKC activity. The translocation of PKC-βI and -δ was blocked by the PLC inhibitor U73122 whereas that of PKC-ζ was abolished by the PI-3 kinase inhibitor wortmannin. The attenuation of cAMP production by $1,25(OH)_2D_3$ was antagonized by the PKC inhibitors Gö6850, calphostin C, and wortmannin, but not by a calmodulin kinase II (CaMKII) inhibitor. Treatment with $1,25(OH)_2D_3$ for 20 min increased AC VI phosphorylation by 10.8-fold and this was blocked partially by Gö6850 and partially by wortmannin but was unaffected by CaMKII inhibitor. These results demonstrate that $1,25(OH)_2D_3$ activation of PKC isoforms leads to phosphorylation of AC VI and inhibition of PTH-activation of this pathway in osteoblasts. J. Cell. Biochem. 94: 1017–1027, 2005.

Key words: parathyroid hormone; 1,25-dihydroxyvitamin D_3; adenylyl cyclase type VI; protein kinase C; phosphatidylinositol-3 kinase; osteoblast

Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are two of the primary calcitropic hormones that work in concert to increase extracellular calcium levels in the face of decreased serum calcium. At the level of the bone, both PTH and $1,25(OH)_2D_3$ increase bone turnover by distinct mechanisms. PTH acts on cell surface receptors on osteoblasts to increase adenylyl cyclase (AC) and phospholipase C (PLC) activity [Offermanns et al., 1996; Mitchell and Bansal, 1997], while $1,25(OH)_2D_3$ mediates its effects on bone primarily by interaction with the nuclear vitamin D receptor (VDR).

Stimulation of AC by PTH has been shown to be the primary mechanism by which it regulates osteoblastic activity. Increasing cAMP results in increases in synthesis and secretion of collagenase-3 (matrix metalloproteinase 13) [Winchester et al., 1999] as well as the receptor activator of nuclear factor-κB ligand (RANKL) [Lee and Lorenzo, 1999]. Both of these actions are required for PTH-mediated bone resorption. The role of the PLC system in mediating PTH actions in bone is less clear. Several studies suggest that PTH activation of protein kinase C (PKC), via the stimulation of PLC, plays a role in stimulating the synthesis and release of transforming growth factor-β1 (TGF-β1) [Wu and Kumar, 2000] and we have shown that it plays a

Grant sponsor: Canadian Institutes for Health Research.

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Received 13 May 2004; Accepted 7 October 2004

DOI 10.1002/jcb.20366

significant role in PTH-stimulation of the synthesis of insulin-like growth factor binding protein-5 (IGFBP-5) [Erclik and Mitchell, 2002]. Both of these factors stimulate bone deposition by activating osteoblast growth and differentiation and may, therefore, play a role in the coupling of bone resorption to bone deposition.

The $1,25(OH)_2D_3$–VDR complex combined with retinoid X receptors binds to vitamin D response elements (VDRE) in the promoter regions of a number of genes resulting in changes in osteoblastic gene expression [Haussler et al., 1998]. $1,25(OH)_2D_3$ increases the expression of osteocalcin, osteopontin, alkaline phosphatase, and RANKL.

In addition to the nuclear receptors, many studies have demonstrated the effects of $1,25(OH)_2D_3$ on PKC isoforms in a number of different cell lines including rat osteoblasts [van Leeuwen et al., 1992; Boyan et al., 2002; Rivera-Bermudez et al., 2002]. It is not clear how $1,25(OH)_2D_3$ regulates PKC activity in these cells. However, several studies have shown that it occurs independent of the nuclear VDR and may be mediated by interaction with a putative membrane receptor [Nemere et al., 1998; Baran et al., 2000; Wali et al., 2003]. It is also not clear what effect if any the regulation of PKC by $1,25(OH)_2D_3$ has on osteoblast activity.

Although PTH and $1,25(OH)_2D_3$ act synergistically to increase extracellular calcium, they also limit each others effects at a number of levels. $1,25(OH)_2D_3$ inhibits PTH synthesis in the parathyroids, mediated by an inhibitory VDRE within the PTH gene [Silver et al., 1985]. At the level of bone, PTH has been shown to inhibit the synthesis of VDR [Reinhardt and Horst, 1990] and $1,25(OH)_2D_3$ has been shown to inhibit PTH-stimulated AC activity [Chen and Feldman, 1984; Catherwood, 1985; Kubota et al., 1985]. The inhibition of AC by $1,25(OH)_2D_3$ appears to occur at multiple levels. Following prolonged stimulation by $1,25(OH)_2D_3$, PTH receptors are down-regulated [Xie et al., 1996], but there is also a more rapid effect of $1,25(OH)_2D_3$ that may involve other components of the AC system.

In the present study, we investigated the mechanism by which $1,25(OH)_2D_3$ inhibited PTH and forskolin-mediated AC response in osteoblastic UMR 106-01 cells. We found that $1,25(OH)_2D_3$ stimulated the membrane translocation and activation of PKC-βI, -δ, and -ζ and using different protein kinase inhibitors we found that PKC isoforms are involved in the $1,25(OH)_2D_3$-mediated inhibition of AC activity. Using RT-PCR, we determined the predominant AC subtype present in UMR cells to be AC VI and we demonstrated that $1,25(OH)_2D_3$ treatment could phosphorylate AC VI by activating PKC.

MATERIALS AND METHODS

Materials

Rat PTH (1–34) was purchased from Bachem Bioscience (King of Prussia, PA). $1,25(OH)_2D_3$, $25(OH)D_3$, U73122, and wortmannin were from Biomol (Plymouth Meeting, PA). Pansorbin, Gö6850, calphostin C, and CaMKII inhibitor 281–309 were purchased from Calbiochem (San Diego, CA). AC VI antibody was from Santa Cruz (Santa Cruz, CA) and isoform-specific PKC antibodies were purchased from BD Transduction Laboratories (Mississauga, Ont., Canada). [^{32}P]ortho-phosphate and [α-^{32}P] ATP were purchased from ICN (Aurora, OH). Trizol, reverse transcriptase, TA cloning vector pCR 2.1, DH5α compentent cells and all cell culture media were purchased from Invitrogen (Oakville, Ont., Canada). [γ-^{32}P]ATP and ECL reagents were from Amersham Biosciences (Baie d'Urfé, Que., Canada).

Cell Culture

UMR 106-01 rat osteosarcoma cells (a gift from Dr. N. Partridge, University of Medicine and Dentistry of New Jersey, Piscataway, NJ) were maintained in 50% Dulbecco's modified Eagle's medium: 50% Ham's F12 (50:50) culture medium supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B, and 5% (vol./vol.) fetal bovine serum. Cells were grown at 37°C in a humidified 95% air, 5% CO_2 atmosphere. Experiments were carried out on cells between 21st and 30th passage.

AC Activity Assay

AC activity was performed as previously described [Jacobowitz et al., 1994]. Cells were plated at a density of 4×10^5 cells/cm^2 on 24-well plates and pre-incubated with $1,25(OH)_2D_3$, or $25(OH)D_3$ the next day for the times indicated in individual experiments. The cells were washed with hypotonic buffer (10 mM HEPES, 2 mM EDTA, 0.1 mM AEBSF) and then lysed in situ by

adding 100 μl of hypotonic buffer and incubated at 37°C for 15 min. Cells were then stimulated by addition of 10 nM PTH, 100 μM forskolin, or control vehicle to the medium in the presence of assay buffer (30 mM HEPES, 2 mM EDTA, 0.1 mM AEBSF, 0.5 mM IBMX, 20 mM creatine phosphate, 10 μg/ml creatine kinase, 12 mM $MgCl_2$, 0.1 mM [α-^{32}P] (~3,000 cpm/pmol) ATP) followed by incubation at 37°C for 15 min. The assay was terminated by the addition of a stop solution (1% SDS, 40 mM ATP, 10 mM cAMP). The [^{32}P] cAMP formed was isolated by the method of Salomon et al. [1974] and quantified using a β counter.

RT-PCR of AC Subtypes

Total RNA was isolated from UMR cells using Trizol Reagent protocol. Total RNA (1 μg) was reverse-transcribed in 20 μl reaction mixture containing 0.2 μg random hexamer, 0.01 M DTT, 0.5 mM dNTPS, 38.5 U RNA guard and 1 U of reverse transcriptase. The mixture was incubated for 1 h at 37°C. PCR was performed using the generated cDNA and primers designed to amplify all nine subtypes of AC. The degenerate oligonucleotide primers based on conserved regions of rat ACs were sense primer 5′-(C,T)T(A,C,G) GG(A,C,G,T) GA(C,T) TG(C,T) TA(C,T) TAC TG-3′ and anti-sense primer 5′-(A,G)GA CCA (C,G,T)AC (A,G)TC (A,G)(A,T)A (C,T)TG CCA-3′, as described previously [Krupinski et al., 1992]. PCR was performed using 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C with a final extension time of 5 min at 72°C. A PCR product of 212 bp was purified from a 2% agarose gel and subcloned into a TA cloning vector pCR 2.1. DH5α cells were transformed with the vector and ampicillin-resistant colonies selected. Miniprep extracts were then sequenced (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ont., Canada) and the nucleotide sequences were identified by Blast search of the GeneBank database.

Subcellular Fractionation and Western Blot Analysis

Cells were plated at a density of 4×10^5 cells/cm^2 in P100 dishes. After the 1,25 (OH)$_2$D$_3$ treatment, cells were harvested and cytosol, plasma membrane and nuclear fractions were separated according to a previously published protocol [Erclik and Mitchell, 2002]. Protein concentrations were determined by the Amido Black Method [Schaffner and Weissmann, 1973]. Equal amounts (10 μg) of protein from each fraction was resolved on 8% acrylamide gels as described previously [Mitchell and Bansal, 1997]. The blots were blocked with 3% BSA in PBST (PBS + 0.2% Triton) and then probed with either monoclonal antibodies for PKC-α, -δ, -ε, and -ι, or polyclonal antibodies for PKC-βI, -βII, and -ζ, using concentrations of 1:5,000 for -α; 1:1,000 for -ε and -ι; 1:500 for -βI and -δ; 1:200 for -βII and -ζ. The blots were then washed and incubated with the appropriate secondary antibodies conjugated to horseraddish peroxidase. The blots were further washed and subsequently incubated with ECL reagents.

PKC Activity Assay

Cells were subcultured into 100 mm petri dishes and grown to confluence. After treatment with 1,25(OH)$_2$D$_3$ in serum-free media containing 0.1% BSA, cells were washed once with ice-cold PBS and lysed in lysis buffer (50 mM Tris pH 7.4, 0.5 mM EGTA, 1 mM DTT, 2 mM AEBSF, 1 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml trypsin inhibitor). Cells were allowed to swell in buffer for 30 min or until fully lysed. The lysate was centrifuged at 600*g* for 20 min at 4°C to remove nuclei. The supernatant was then re-centrifuge at 100,000*g* for 30 min at 4°C to separate the membrane and cytosol fractions. The membrane pellet was solublized in lysis buffer containing 1% NP-40 and 0.3% deoxycholate. PKC-βI and -δ were immunoprecipitated from the lysates overnight at 4°C using subtype-specific antibodies and Pansorbin. Immunoprecipitated complexes were washed twice with lysis buffer and twice with kinase wash buffer (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 2.1 mM $CaCl_2$). Pelted immunocomplexes were resuspended in 50 μl of kinase wash buffer and then used for kinase assays. In vitro kinase activity was assayed as described [Yasuda et al., 1990] by incubating immunocomplexed PKC in kinase assay buffer (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 2.1 mM $CaCl_2$, 1 μg/ml diolein, 10 μg/ml phosphatidylserine, 25 μM myelin basic protein, 10 μM ATP, and 10μCi γ[^{32}P]-ATP) at room temperature for 40 min. Non-specific activity was determined by the same method in the presence of 70 μM PKC inhibitor peptide 19–31. Following incubation, reactions were terminated by centrifugation at

2,000*g* for 30 s. Supernatants were then spotted onto phospho-membranes and washed three times in 1% phosphoric acid. Dried membranes with bound ^{32}P were then quantitated in a scintillation counter.

In Vivo Phosphorylation of AC VI

Phosphorylation experiments was performed according to previously published methods [Varga et al., 1999]. One million cells were plated in 100 mm petri dishes and grown in 50:50 medium with 5% FBS for 2 days. Cells were then incubated in phosphate-free DMEM supplemented with 10% FBS for 2 h and 0.25 mCi/ml [^{32}P] ortho-phosphate was added to label the cells for another 2 h. Stimulation was carried out during the last 20 min of incubation by addition of 100 nM $1,25(OH)_2D_3$ with or without 5 μM Gö6850, 10 nM wortmannin, or 100 nM CaMKII inhibitor. Some cells were incubated with 1 μM PMA, a non-selective PKC stimulator. Cells were washed twice with phosphate-free DMEM and scraped into ice-cold homogenization buffer. Cell lysates were centrifuged, resuspended in RIPA buffer and incubated on ice for 1 h. After centrifugation, the supernatants were pre-cleared with rabbit pre-immune serum and Pansorbin for 1 h at 4°C then incubated with 6 μg of AC VI specific antibody for 1 h. Pansorbin was then added and the mixture was incubated overnight at 4°C with gentle rocking. The beads were washed twice with homogenization buffer and the immunoprecipitates eluted from the beads by incubating with Laemmli sample buffer at 65°C for 5 min and resolved on 8% denaturing polyacrylamide gels. The gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography.

Data Presentation

The data presented are representative of experiments performed at least three times. The values plotted in bar graphs are the means of three individual experiments ± SE. Statistical significance was evaluated by Student's *t*-test.

RESULTS

Effect of $1,25(OH)_2D_3$ on AC Activity in UMR 106-01 Cells

In order to examine the short-term effect $1,25(OH)_2D_3$ on PTH-mediated AC activity, UMR cells were pre-incubated with various concentrations of $1,25(OH)_2D_3$ for 20 min and cAMP production in response to 10 nM PTH or 100 μM forskolin were measured. As shown in Figure 1A, $1,25(OH)_2D_3$ inhibited both PTH and

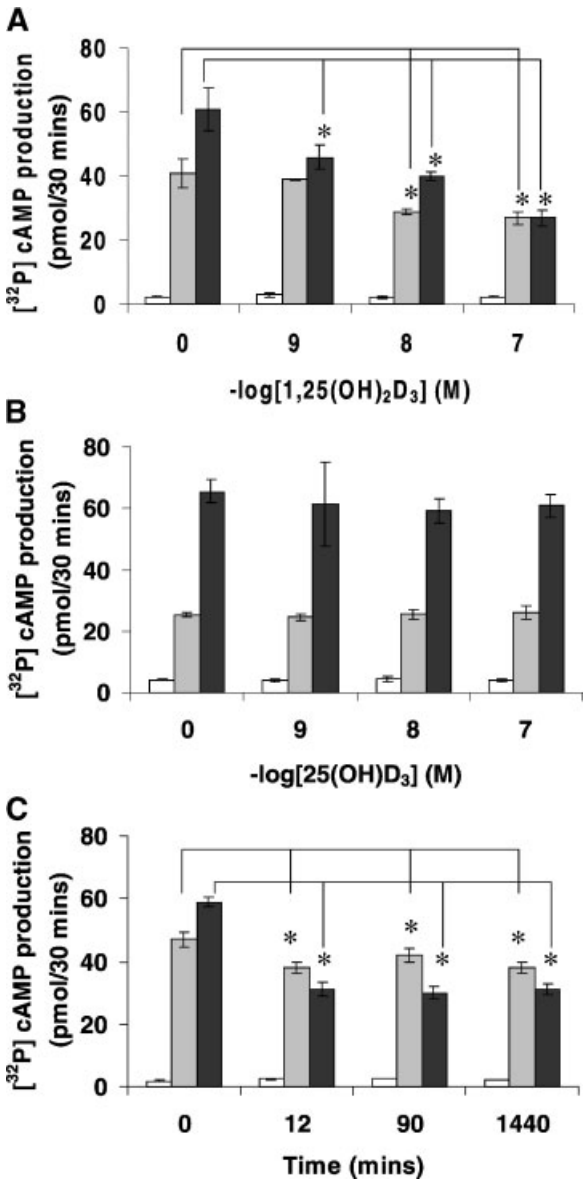

Fig. 1. Characterization of $1,25(OH)_2D_3$ effects on adenylyl cyclase (AC) activity in UMR cells. Concentration dependence of $1,25(OH)_2D_3$ (**A**), $25(OH)D_3$ (**B**), or time dependence of $1,25(OH)_2D_3$ (**C**) on basal, parathyroid hormone (PTH) and forskolin-stimulated AC activity. UMR cells were pre-treated with 0.1% ethanol (control vehicle) or indicated concentrations of either $1,25(OH)_2D_3$ or $25(OH)D_3$ for 20 min (for concentration effect experiment) or 100 nM $1,25(OH)_2D_3$ for indicated times (for time course experiment). Cells were then lysed and stimulated with control vehicle (white bars), 10 nM (for A and C) or 1 nM (for B) PTH (gray bars), 100 μM forskolin (black bars) for 15 min. Values are mean ± SE from three independent experiments (n = 3). *, $P < 0.05$ versus control.

forskolin-mediated cAMP production in a concentration-dependent manner from 25% to 50% while the basal cyclase activity remained unchanged. When cells were incubated with the same concentrations of the inactive metabolite 25(OH)D$_3$ there was no effect on AC activity in response to either 1 nM PTH or 100 μM forskolin (Fig. 1B). The inhibitory effect of 1,25(OH)$_2$D$_3$ was already maximal after 12 min and sustained over 24 h (Fig. 1C).

Identification of AC Subtypes Expressed in UMR 106-01 Cells

The inhibition of both PTH and forskolin-stimulated cAMP production by 1,25(OH)$_2$D$_3$ treatment suggested that the AC enzyme was the primary target of regulation by the secosteroid. Since each AC subtype has different regulatory properties, it is important to determine the profile of AC subtypes expressed in UMR cells. Using degenerate oligonucleotide primers corresponding to conserved sequence in the first intracellular loop of AC, PCR products were amplified from cDNA prepared from UMR cells. No products were detected in control experiments using samples generated in the absence of reverse transcriptase. PCR products were sub-cloned into pCR 2.1 vectors and sequenced. Sequence analysis of 50 randomly selected clones revealed AC III, VI, VII, VIII in UMR cells (Table I). RT-PCR using subtype-specific primers confirmed the expression of these AC subtypes in UMR cells. Type VI AC was shown to be the major form expressed in UMR cells, constituting 50% of the 50 isolated clones. AC type I, II, IV, V, and IX were not detected. These results are the first indication that AC VI mRNA is the predominant cyclase subtype present in cells with osteoblastic phenotype.

Effect of 1,25(OH)$_2$D$_3$ on Translocation and Activity of PKC Isoforms in UMR 106-01 Cells

Since type VI AC has been shown to be the target of regulation by PKC and 1,25(OH)$_2$D$_3$ has been shown to activate PKC in a number of cell types, we examined the ability of 1,25(OH)$_2$D$_3$ to activate PKC in UMR cells. To determine whether 1,25(OH)$_2$D$_3$ could activate PKC isoforms expressed in UMR cells, we examined the subcellular localization of various PKC isoforms by Western blot analysis before and after treatment with 100 nM 1,25(OH)$_2$D$_3$ for 20 min. As shown in Figure 2 and Table II, PKC-α, -βII, -ε, and -ι isoforms were unaffected by 1,25(OH)$_2$D$_3$ treatment, while PKC-βI, -δ, and -ζ were affected by treatment with 1,25(OH)$_2$D$_3$. PKC-βI was predominantly localized to the cytosol with small amounts in the membrane fraction in control cells. Treatment with 1,25(OH)$_2$D$_3$ stimulated the translocation of classic PKC-βI from the cytosol to the membrane, increasing membrane associated PKC-βI from $6.7 \pm 1.8\%$ to $19.7 \pm 0.9\%$ of the total PKC-βI expression. The novel PKC-δ isoform was also stimulated to translocate to the membrane in the presence of 1,25(OH)$_2$D$_3$ with $32.3 \pm 5.6\%$ of PKC-δ in the membrane in control cells and $63 \pm 5.8\%$ associated with the membrane in 1,25(OH)$_2$D$_3$ treated cells. The atypical PKC isoform PKC-ζ was present in all fractions but again treatment with 1,25(OH)$_2$D$_3$ increased the percentage of the total PKC-ζ in

TABLE I. Adenylyl Cyclase (AC) Subtypes Expression in UMR 106-01 Cells

AC subtypes	I	II	III	IV	V	VI	VII	VIII	IX
%	0	0	6	0	0	50	27	17	0

The PCR products were subcloned to pCR 2.1 vector and each clone was identified by DNA sequencing analysis. The values are the percentages of the total 50 clones expressing each AC subtype.

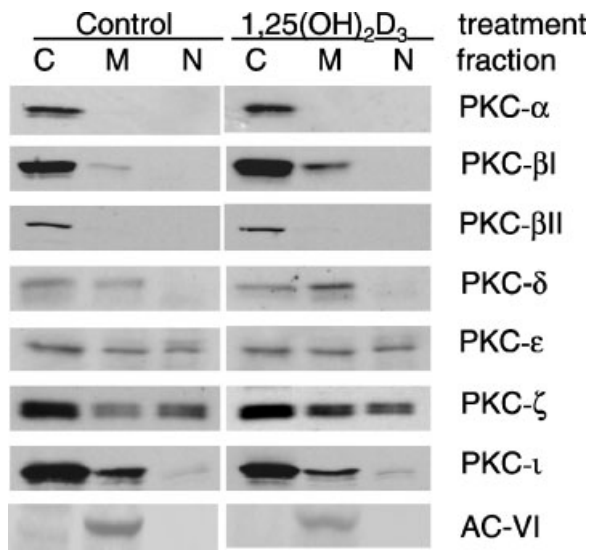

Fig. 2. Regulation of the subcellular distribution of PKC isoforms by 1,25(OH)$_2$D$_3$ in UMR 106-01 cells. Cells were treated with either 0.1% ethanol (control vehicle) or 100 nM 1,25(OH)$_2$D$_3$ for 20 min, and then cytosol (C), membrane (M), and nuclear (N) fractions were prepared and immunoblotted with antibodies specific for PKC-α, -βI, -βII, -δ, -ε, -ζ, -ι, and AC type VI. Blots are representative of three independent experiments.

TABLE II. Quantitative Analysis of the Effects of $1,25(OH)_2D_3$ on Subcellular Distribution of Various PKC Isoforms Expressed in UMR 106-01 Cells

Treatment	Fractions	α (%)	βI (%)	βII (%)	δ (%)	ε (%)	ζ (%)	ι (%)
Control	C	100 ± 0	93.3 ± 1.8	100 ± 0	65.6 ± 2.1	48.7 ± 1.4	72.8 ± 0.9	69.8 ± 2
	M	0 ± 0	6.7 ± 1.8	0 ± 0	32.3 ± 5.6	30.2 ± 1	15.7 ± 6.2	27.5 ± 2.3
	N	0 ± 0	0 ± 0	0 ± 0	2.1 ± 2	21.1 ± 0.8	11.5 ± 6.8	2.7 ± 0.3
$1,25(OH)_2D_3$	C	100 ± 0	80.1 ± 0.7	100 ± 0	35.7 ± 7	37.5 ± 5.2	54.7 ± 4.9	69.1 ± 4
	M	0 ± 0	19.7 ± 0.9**	0 ± 0	63 ± 5.8**	32 ± 3.8	36 ± 10.9*	28.1 ± 2.7
	N	0 ± 0	0.2 ± 0.2	0 ± 0	1.3 ± 1.2	30.5 ± 9	9.3 ± 6.2	2.8 ± 1.3

Quantitative evaluation of data (expressed in percentage of total PKC) from three independent experiments. C, cytosol; M, membrane; N, nuclear.
*$P < 0.05$ versus control.
**$P < 0.001$ versus control.

the membrane fraction from 15.7 ± 6.2% to 36 ± 10.9%. AC type VI immunoreactivity was only found in the membrane fraction from control or $1,25(OH_2)D_3$ treated cells (Fig. 2).

PKC activity was assessed in membrane fractions from UMR cells following immunoprecipitation using isoform-specific antibodies to PKC-βI and PKC-δ. Treatment with 100 nM $1,25(OH)_2D_3$ for 20 min increased the PKC-βI activity in the membrane fraction from 3 ± 0.7% to 18.8 ± 2% of total PKC-βI activity in the cell and PKC-δ activity in the membrane fraction from 12 ± 0.5% to 28.5 ± 7.7% of total PKC-δ activity. The activity of PKC-ζ could not be assessed, as commercially available PKC-ζ antibodies did not immunoprecipitate the enzyme.

The Role of Phospholipase C (PLC) and PI-3 Kinase in $1,25(OH)_2D_3$-Activated PKC-βI, -δ, and -ζ

Our results suggested that $1,25(OH)_2D_3$ could activate PKC-βI and PKC-δ, both DAG-sensitive isoforms, to translocate from cytosol to membrane. In order to determine if $1,25(OH)_2D_3$-induced PKC-βI and -δ translocation via a PLC-dependent pathway, UMR cells were treated with 100 nM of $1,25(OH)_2D_3$ in the presence of 10 μM U73122, a PLC inhibitor, for 20 min. As shown in Figure 3A and Table III, U73122 did not affect the basal distribution of PKC-βI or -δ, but blocked the $1,25(OH)_2D_3$-induced translocation of both isoforms from cytosol to membrane. Treatment with U73122 had no effect on $1,25(OH)_2D_3$-stimulated translocation of PKC-ζ (Fig. 3A). These results suggested that $1,25(OH)_2D_3$-mediated translocation of PKC-βI and -δ required prior activation of the PLC signaling pathway.

Previous studies had indicated that the DAG-insensitive PKC-ζ could be activated by the phosphatidylinositol-3 (PI-3) kinase pathway [Bandyopadhyay et al., 1997; Mendez et al., 1997; Kotani et al., 1998] and this pathway has been shown to be activated by $1,25(OH)_2D_3$ [Rebsamen et al., 2002]. In order to test whether $1,25(OH)_2D_3$-induced membrane translocation of PKC-ζ via PI-3 kinase, UMR cells were exposed for 20 min to 100 nM of $1,25(OH)_2D_3$ with or without 10 nM wortmannin (a PI-3 kinase inhibitor). As shown in Figure 3B and Table III, $1,25(OH)_2D_3$-induced translocation of PKC-ζ to the membrane was blocked by wortmannin while wortmannin itself had little effect on basal distribution of PKC-ζ. Wortmannin was not able to block the translocation of other PKC isoforms by $1,25(OH)_2D_3$ (Fig. 3B).

The Role of PKC in Inhibition of AC Activity by $1,25(OH)_2D_3$

To determine if activation of PKC isoforms by $1,25(OH)_2D_3$ played a role in the observed

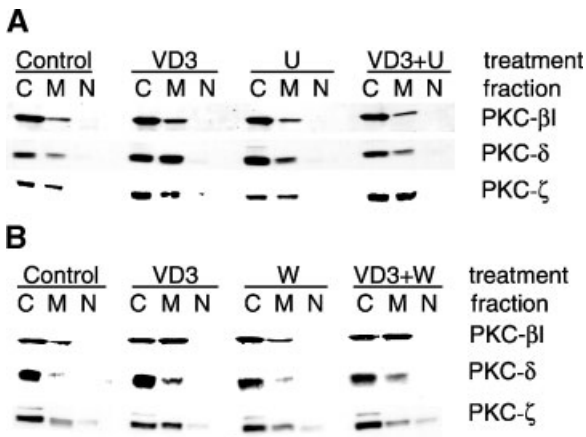

Fig. 3. Effect of the PLC inhibitor U73122 (**A**) and PI-3 kinase inhibitor wortmannin (**B**) on $1,25(OH)_2D_3$-mediated translocation of PKC isoforms. UMR cells were treated for 20 min with 100 nM $1,25(OH)_2D_3$ (VD3), with or without 10 μM U73122 (U) or 10 nM wortmanin (W), and then cytosol (**C**), membrane (M) and nuclear (N) fractions were prepared and immunoblotted with antibodies specific for PKC-βI, -δ, or -ζ. Blots are representative of three independent experiments.

TABLE III. Quantitative Analysis of the Effects of U73122 and Wortmannin on 1,25(OH)$_2$D$_3$-Induced Translocation of PKC-βI, -δ, and -ζ in UMR 106-01 Cells

Isoform	Fractions	Control (%)	1,25(OH)$_2$D$_3$ (%)	U73122 (%)	1,25(OH)$_2$D$_3$ + U73122 (%)
βI	C	88.2 ± 5.4	69.6 ± 1.6	89.4 ± 6.7	89.5 ± 8.6
	M	11.8 ± 5.4	30.2 ± 1.6***	10.6 ± 6.7	10.3 ± 8.8
	N	0 ± 0	0.2 ± 0.2	0 ± 0	0.2 ± 0.2
δ	C	73.5 ± 3.3	37.5 ± 9.6	71.9 ± 12	75.7 ± 1
	M	26 ± 3.1	62 ± 9.2***	27.8 ± 11.8	24.1 ± 1
	N	0.5 ± 0.5	0.5 ± 0.5	0.3 ± 0.3	0.2 ± 0.2
ζ	C	86.1 ± 6.7	64.4 ± 3.6	83.2 ± 8.5	61.1 ± 4.9
	M	13.6 ± 6.8	34.6 ± 4.6*	15.4 ± 7.1	38.1 ± 5.3**
	N	0.3 ± 0.1	1 ± 1	1.4 ± 1.4	0.8 ± 0.7
Isoform	Fractions	Control (%)	1,25(OH)$_2$D$_3$ (%)	Wortmannin (%)	1,25(OH)$_2$D$_3$ + Wortmannin (%)
βI	C	87.7 ± 0.5	65 ± 7.9	89.1 ± 2.4	63.4 ± 2.8
	M	12.3 ± 0.5	34.9 ± 8**	10.9 ± 2.4	35.7 ± 3.7****
	N	0 ± 0	0.1 ± 0.1	0 ± 0	0.9 ± 0.9
δ	C	88.1 ± 1	71.7 ± 2.1	90.2 ± 1.4	70.8 ± 2.2
	M	11.4 ± 1.3	28.2 ± 2.2****	9.6 ± 1.3	28.5 ± 1.8****
	N	0.5 ± 0.5	0.1 ± 0.1	0.2 ± 0.2	0.7 ± 0.6
ζ	C	89.2 ± 9.4	63.2 ± 5.8	88.1 ± 10.9	86.5 ± 7.2
	M	10.1 ± 9.9	36.3 ± 6*	11.6 ± 10.8	12.7 ± 7.9
	N	0.7 ± 0.5	0.5 ± 0.4	0.3 ± 0.2	0.8 ± 0.7

Quantitative evaluation of data (expressed in percentage of total PKC) from three independent experiments. C, cytosol; M, membrane; N, nuclear.
*$P < 0.05$ versus control.
**$P < 0.01$ versus control.
***$P < 0.001$ versus control.
****$P < 0.0005$ versus control.

inhibition of PTH and forskolin-stimulated AC by 1,25(OH)$_2$D$_3$, UMR cells were treated with 100 nM 1,25(OH)$_2$D$_3$ for 20 min in the presence of the PKC inhibitors Gö6850 (1 μM), calphostin C (100 nM), or the PI-3 kinase inhibitor wortmannin (10 nM). 1,25(OH)$_2$D$_3$-induced inhibition of PTH and forskolin stimulated cAMP production were blocked by both Gö6850 and wortmannin but not by a CaMKII inhibitor (Fig. 4), suggesting that activation of PKC pathways was required for the regulation of AC activity by 1,25(OH)$_2$D$_3$.

Stimulation of AC VI Phosphorylation by 1,25(OH)$_2$D$_3$ in UMR 106-01 Cells

Since type VI AC was the major subtype expressed in UMR cells and its activity was inhibited by 1,25(OH)$_2$D$_3$ stimulation of PKC activity, we assessed whether 1,25(OH)$_2$D$_3$ could inhibit AC VI expression or stimulate its phosphorylation. The expression of AC VI mRNA was measured by semi-quantitative RT-PCR with subtype-specific primers and AC VI protein was examined by Western blot using subtype-specific antibody. Neither AC VI mRNA nor protein expression were altered by 1,25(OH)$_2$D$_3$ treatment as shown in Figure 5.

Metabolic labeling and immunoprecipitation were used to measure the incorporation of ^{32}P into AC VI protein. Two bands of phosphorylated protein migrating at approximately 130 and 200 kDa were observed on the autoradiograph, which correspond to the deduced molecular weight of AC VI and its glycosylated form, respectively. Since the density of the two bands measured for each treatment was very similar, we used the 130 kDa band to quantitate phosphorylation. As shown in Figure 6, in the absence of any treatment, ^{32}P incorporation into the 130 kDa protein was very low. Treatment of

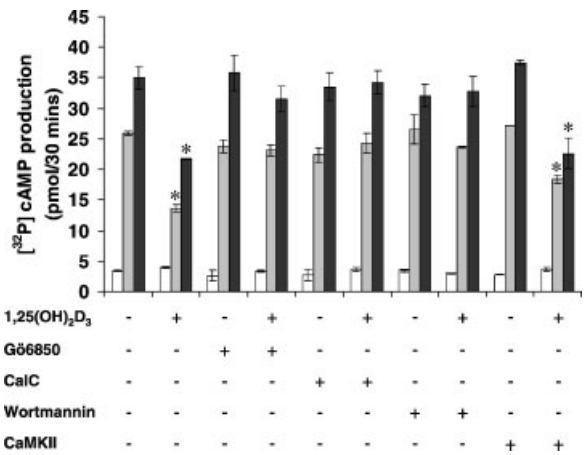

Fig. 4. Effect of the PKC, PI-3 kinase, and CaMKII inhibitors on 1,25(OH)$_2$D$_3$-mediated inhibition of AC activity in UMR cells. Cells were pre-treated with 0.1% ethanol (control vehicle) or 100 nM 1,25(OH)$_2$D$_3$ with or without 1 μM Gö6850, 100 nM calphostin C (CalC), 10 nM wortmannin, or 100 nM calcium-calmodulin kinase II (CaMKII) inhibitor for 20 min. Cells were then lysed and stimulated with control vehicle (white bars), 10 nM PTH (gray bars), 100 μM forskolin (black bars) for 15 min. Values are mean ± SE from three independent experiments (n = 3). *, $P < 0.01$ versus control.

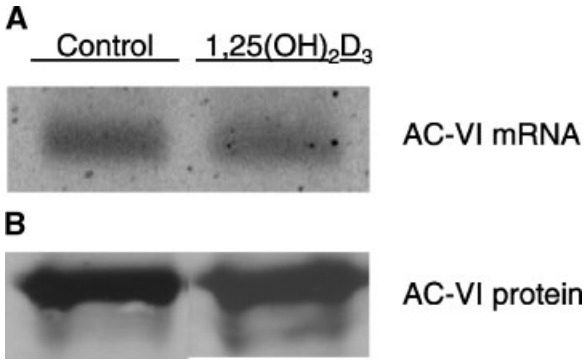

Fig. 5. Characterization of $1,25(OH)_2D_3$ effects on AC (**A**) mRNA and (**B**) protein expression in UMR cells. Cells were treated with either 0.1% ethanol (control vehicle) or 100 nM $1,25(OH)_2D_3$ for 20 min, and then total RNA or whole cell lysate was collected. A: Semi-quantitative RT-PCR was performed using AC VI-specific primers and PCR products (230 bp) were resolved in 1.5 % agarose gel. B: For Western blot analysis, cell lysate (80 μg) was resolved on 8% SDS–PAGE, transferred to nitrocellulose and immunoblotted with antibody specific for AC VI (130 kDa). Blots are representative of three independent experiments.

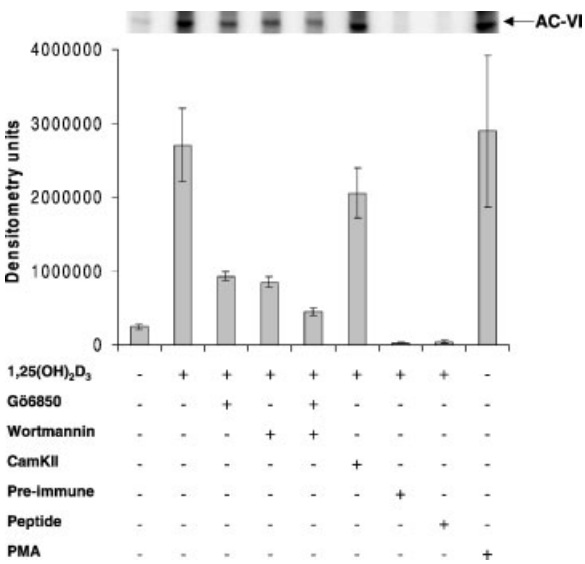

Fig. 6. In vivo phosphorylation of AC VI in UMR 106-01 cells. Cells were phosphate-deprived for 2 h and then radiolabeled with 0.2 mCi/ml [^{32}P] ortho-phosphate for another 2 h. Treatment were carried out at the last 20 min of incubation with 0.1% ethanol (control vehicle), 1 μM PMA (positive control), 100 nM $1,25(OH)_2D_3$ with or without 1 μM Gö6850, 10 nM wortmannin and 100 nM CaMKII inhibitor. The cell lysate was incubated with 10% Pansorbin and AC VI specific-antibody, with or without the blocking peptide, or pre-immune serum. The immunoprecipitates were resolved in 8% SDS-PAGE, then stained, dried, and exposed to phosphorimager. The quantitation of [^{32}P] incorporation was determined by scanning densitometry. Values are mean ± SE from three independent experiments (n = 3).

the cells with 100 nM $1,25(OH)_2D_3$ for 20 min significantly increased the phosphorylation of AC VI by about 10.8-fold. A similar magnitude of phosphorylation of AC VI was also seen when the cells were treated with the non-specific PKC activator PMA. Treatment of the cells with $1,25(OH)_2D_3$ in the presence of Gö6850 or wortmannin decreased the cyclase VI phosphorylation by approximately 65% and co-treatment with both inhibitors led to further attenuation of the $1,25(OH)_2D_3$-mediated AC VI phosphorylaton. Incubation with the CaMKII inhibitor on the other hand had no effect on $1,25(OH)_2D_3$-mediated AC VI phosphorylation. The total amounts of AC VI present in each immunoprecipitate were comparable and varied by less than 10% when assessed by Western blot analysis (data not shown). To demonstrate specificity of the phosphorylation signal in the assay, addition of excess blocking peptide to the immunoprecipitation reaction or immunoprecipitation with pre-immune serum were included and showed no significant levels of phosphorylated protein.

These results demonstrated that phosphorylation of AC VI by $1,25(OH)_2D_3$ was mediated by PKC and PI-3 kinase pathways. Our data are the first to demonstrate phosphorylation of AC and a role for $1,25(OH)_2D_3$ activation of PKC isoforms in osteoblastic cells.

DISCUSSION

There are a number of well characterized mechanisms by which cellular signals cross regulate in order for cells to respond appropriately to multiple endocrine signals. The aim of this study was to determine the mechanism by which $1,25(OH)_2D_3$ modulates PTH activation of AC, its primary signal transduction system. As found by others, $1,25(OH)_2D_3$ treatment could inhibit PTH stimulation of AC, but we also noted that forskolin-stimulated cAMP production was inhibited to a similar extent suggesting that the short-term regulatory effects of $1,25(OH)_2D_3$ are at the level of the cyclase enzyme itself. In order to understand this further, we examined which AC subtypes were expressed in our cells. Like many other cell types, the UMR cells express multiple subtypes of AC and we identified four by our screening method. In the absence of purified cyclase proteins, it is not possible to assess the levels of expression of each subtype in a cell and so

we followed the approach used previously [Krupinski et al., 1992; Takahashi et al., 1998] in which degenerate oligonucleotide primers are used to amplify one of the highly homologous regions in all nine subtypes of cyclase and then by sequencing a random selection of PCR products the ratio and identity of the subtypes could be established. The results indicated that UMR cells expressed four different cyclase subtypes, AC III, VI, VII, and VIII with AC VI the major subtype constituting 50% of all the sequenced clones. Type VI cyclase has been shown to be expressed in many tissues [Yoshimura and Cooper, 1992], and can be inhibited by $G_i\alpha$, calcium, PKA, and PKC. In particular, the PKC-mediated inhibition and phosphorylation of AC VI have been well characterized [Chern et al., 1995; Lai et al., 1997]. Deletion and mutation studies have shown that several serine and threonine residues of AC VI are important for its inhibition and phosphorylation by PKC [Lai et al., 1999; Lin et al., 2002]. The other two AC subtypes expressed to significant levels in the UMR cells were type VII and type VIII. Expression of type VII AC in HEK293 cells was stimulated by phorbol esters suggesting that PKC isozymes may phosphorylate this adenylyl cyclase subtype [Watson et al., 1994]. We have not investigated if the type VII AC expressed in UMR cells was phosphorylated following treatment with 1,25(OH$_2$)D$_3$, and it is unlikely to have affected the results that we saw in the AC assays as AC VII is very rapidly but transiently activated by PKC with responses returning to near control levels within 7 min of PKC stimulation [Watson et al., 1994]. AC type VIII has not been reported to be affected by PKC and thus is unlikely to contribute to the effects that we have seen of 1,25(OH$_2$)D$_3$ on AC activity in the UMR cells.

Previous studies have implicated PKC as a component of the signaling pathway of 1,25(OH)$_2$D$_3$, which led us to study the activation of PKC isoforms by 1,25(OH)$_2$D$_3$ in UMR osteoblastic cells as a potential mechanism of regulating AC activity. We found three PKC isoforms, -βI, -δ, and -ζ, were translocated to the cell membrane in response to 1,25(OH)$_2$D$_3$ stimulation with concomitant increases in PKC activity suggesting they could be responsible for AC VI inhibition by phosphorylating the enzyme. Indeed the amount of radiolabeled ^{32}P incorporated into immunoprecipitated AC VI was significantly increased by 1,25(OH)$_2$D$_3$ stimulation and this was blocked by PKC inhibitors. Together with the data showing that 1,25(OH)$_2$D$_3$ inhibition of AC activity could be blocked by PKC inhibitors, this is compelling evidence that the mechanism by which 1,25(OH)$_2$D$_3$ inhibits PTH signal transduction in UMR cells is through PKC phosphorylation of AC VI.

Our data suggest that 1,25(OH)$_2$D$_3$-mediated translocation of PKC-βI and δ, both DAG-sensitive isoforms, occurred via a PLC-dependent pathway. This agreed with previous work suggesting 1,25(OH)$_2$D$_3$ increased total PKC activity by PLC activation in chondrocytes [Schwartz et al., 2001] and short-term treatment of 1,25(OH)$_2$D$_3$ increased IP$_3$, DAG, and intracellular calcium production in ROS 17/2.8 osteosarcoma cells [Civitelli et al., 1990] and rat colonic epithelium [Wali et al., 1990]. We also showed that the translocation of PKC-ζ by 1,25(OH)$_2$D$_3$ was mediated via the PI-3 kinase pathway. 1,25(OH)$_2$D$_3$ has been documented to activate the PI-3 kinase pathway in various other cell types [Hmama et al., 1999; Sly et al., 2001; Rebsamen et al., 2002; Johansen et al., 2003] and our results suggest that this also occurs in osteoblastic cells. Several studies have suggested that PKC-ζ could be a downstream target of PI-3 kinase [Bandyopadhyay et al., 1997; Mendez et al., 1997; Kotani et al., 1998; Liu et al., 1998] and this appears to be the pathway in osteoblasts. As inhibitors of any of the PKC isoforms were sufficient to block 1,25(OH)$_2$D$_3$-mediated phosphorylation of AC VI, we postulate that multiple sites of phosphorylation exist and more than one must be phosphorylated for inhibition to occur. This model is supported by recent evidence showing three sites of PKC phosphorylation on AC VI and phosphorylation of two of these sites are required for inhibition [Lin et al., 2002]. Therefore, it is possible that activation of multiple PKC isoforms by 1,25(OH)$_2$D$_3$ is required to achieve inhibition of AC activity as we observe in our cells.

Although, we have no direct evidence that the inhibition of AC activity by 1,25(OH)$_2$D$_3$ is mediated by cell membrane binding proteins, the time course of inhibition by 1,25(OH)$_2$D$_3$ (observed as early as 12 min) makes it unlikely that regulation of gene transcription is involved. We also saw no changes in either AC VI mRNA levels or protein expression following

$1,25(OH)_2D_3$ treatment, indicating $1,25(OH)_2D_3$ did not alter AC VI expression or degradation. However, the mechanism by which $1,25(OH)_2D_3$ activates PKC in the UMR cells remains to be determined.

While the activation of PKC by $1,25(OH)_2D_3$ has now been demonstrated in many cell types and shown, at least in one study, to be independent of the nuclear VDR, the effects of this activation have not been determined. This is the first report of $1,25(OH)_2D_3$ stimulation of AC VI phosphorylation and opens the possibility that this may be important in other tissues where $1,25(OH)_2D_3$ regulates PTH signal transduction. For example, $1,25(OH)_2D_3$ is known to feedback and inhibit 1α-hydroxylase activation by PTH in the renal proximal tubule [Jones et al., 1998]. As AC VI is the major AC subtype expressed in the proximal tubule [Bek et al., 2001], it is possible that the effect could be at least partially mediated by $1,25(OH)_2D_3$-stimulated PKC phosphorylation of type VI cyclase. In addition to the longer term down-regulation of PTH receptor gene expression by $1,25(OH)_2D_3$, this cross-regulation of AC as we have shown here offers a rapid means of limiting the actions of PTH through its major signal transduction pathway.

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