



Signaling components of the $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent Pdia3 receptor complex are required for Wnt5a calcium-dependent signaling



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ABSTRACT

Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ are important regulators of endochondral ossification. In osteoblasts and growth plate chondrocytes, $1\alpha,25(\text{OH})_2\text{D}_3$ initiates rapid effects via its membrane-associated receptor protein disulfide isomerase A3 (Pdia3) in caveolae, activating phospholipase A₂ (PLA₂)-activating protein (PLAA), calcium/calmodulin-dependent protein kinase II (CaMKII), and PLA₂, resulting in protein kinase C (PKC) activation. Wnt5a initiates its calcium-dependent effects via intracellular calcium release, activating PKC and CaMKII. We investigated the requirement for components of the Pdia3 receptor complex in Wnt5a calcium-dependent signaling. We determined that Wnt5a signals through a CaMKII/PLA₂/PGE₂/PKC cascade. Silencing or blocking Pdia3, PLAA, or vitamin D receptor (VDR), and inhibition of calmodulin (CaM), CaMKII, or PLA₂ inhibited Wnt5a-induced PKC activity. Wnt5a activated PKC in caveolin-1-silenced cells, but methyl-beta-cyclodextrin reduced its stimulatory effect. $1\alpha,25(\text{OH})_2\text{D}_3$ reduced stimulatory effects of Wnt5a on PKC in a dose-dependent manner. In contrast, Wnt5a had a biphasic effect on $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated PKC activation; 50 ng/ml Wnt5a caused a 2-fold increase in $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated PKC but higher Wnt5a concentrations reduced $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated PKC activation. Western blots showed that Wnt receptors Frizzled2 (FZD2) and Frizzled5 (FZD5), and receptor tyrosine kinase-like orphan receptor 2 (ROR2) were localized to caveolae. Blocking ROR2, but not FZD2 or FZD5, abolished the stimulatory effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on PKC and CaMKII. $1\alpha,25(\text{OH})_2\text{D}_3$ membrane receptor complex components (Pdia3, PLAA, caveolin-1, CaM) interacted with Wnt5a receptors/co-receptors (ROR2, FZD2, FZD5) in immunoprecipitation studies, interactions that changed with either $1\alpha,25(\text{OH})_2\text{D}_3$ or Wnt5a treatment. This study demonstrates that $1\alpha,25(\text{OH})_2\text{D}_3$ and Wnt5a mediate their effects via similar receptor components and suggests that these pathways may interact.

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1. Introduction

Endochondral ossification encompasses multiple events during which the embryonic cartilaginous template of long bones is gradually calcified and replaced by bone [1]. Previous reports by our lab and other groups have suggested that a complex network of interacting signaling pathways induced by hormones and growth factors also regulate post-natal growth plate chondrocytes and endochondral bone formation [2–5]. However, limited information is available on the molecular basis of these interactions.

In the present study, we focus on inter-relation between two pathways involved in regulation of post-natal growth plate cartilage and osteoblast maturation: $1\alpha,25$ -dihydroxyvitamin D₃ [$1\alpha,25(\text{OH})_2\text{D}_3$]

membrane-mediated signaling and non-canonical Wnt5a calcium-dependent signaling. $1\alpha,25(\text{OH})_2\text{D}_3$ induces its effects via two mechanisms: classical vitamin D receptor (VDR) signaling and the more recently described calcium-dependent membrane-mediated pathway [6–8]. In the membrane-mediated pathway, $1\alpha,25(\text{OH})_2\text{D}_3$ initiates its effects via its specific membrane-associated receptor protein disulfide isomerase family A, member 3 (Pdia3, also known as ERp60, ERp57, Grp58, and 1,25-MARRS) [9,10] located in caveolae, which are 50–100 nm lipid rafts highly enriched with cholesterol and glycosphingolipids [11]. Caveolae are characterized by caveolin coat proteins (Cav-1, Cav-2, Cav-3) that serve as signaling platforms for several steroid hormones [11]. Upon binding to Pdia3, $1\alpha,25(\text{OH})_2\text{D}_3$ induces interactions between Pdia3 and phospholipase-A₂ (PLA₂)-activating protein (PLAA) [12], stimulating increased calcium/calmodulin-dependent protein kinase II (CaMKII) activity in costochondral growth zone chondrocytes (GC) and MC3T3-E1 osteoblast-like cells [13]. PLA₂ is activated [14], producing lysophospholipid and releasing arachidonic acid (AA) [15] that is further processed into prostaglandin E₂ (PGE₂).

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AA can activate PKC directly [15]. The PGE₂ acts via its EP1 receptor to increase cyclic AMP [16], which results in downstream PKC and ERK1/2 activation [17]. Together with Gαq, lysophospholipid activates phosphatidylinositol-specific phospholipase C beta (PLCβ), generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) [14, 15]. DAG binds PKCα, recruiting it to the plasma membrane [18]. IP3 activates the release of calcium ions required for PKCα activation from the endoplasmic reticulum.

1α,25(OH)₂D₃ stimulates differentiation of growth zone chondrocytes and osteoblasts, increasing alkaline phosphatase specific activity [16] and in the case of osteoblasts, by increasing production of osteopontin [9]. While the VDR mediates many of the effects of 1α,25(OH)₂D₃ on these cells, Pdia3-dependent signaling has been shown to mediate the effects of the vitamin D metabolite on these two markers of osteoblast differentiation. Pdia3 gene knockout results in embryonic lethality [19,20] and as a result, a definitive demonstration of its role has not been possible. However, conditional knockout of Pdia3 in the intestinal epithelium results in severely blunted Ca²⁺ uptake in response to 1α,25(OH)₂D₃ [21], confirming its involvement in 1α,25(OH)₂D₃ dependent actions. Moreover, Pdia3^{+/-} mice exhibit a long bone phenotype [19], further demonstrating the importance of this 1α,25(OH)₂D₃ receptor in skeletal development.

Wnts are signaling molecules that also regulate skeletal development and maintenance [22,23]. In the growth plate, *Wnt5a* expression is low in the reserve zone, increased in proliferative and prehypertrophic zones, and decreased in the hypertrophic region [24]. *Wnt5a* plays an important role in transition of chondrocytes between growth plate zones. Growth plates of *Wnt5a*^{-/-} mice exhibit delayed chondrocyte hypertrophy, suggesting that *Wnt5a* plays a critical role in promoting hypertrophy [25]. *Wnt5a* is also expressed by osteoblasts at the interface between calcified cartilage and metaphyseal bone and it has been shown to promote osteoblast maturation in vitro [5,26]. Osteoblasts isolated from *Wnt5a*^{-/-} mice exhibit down-regulation of osteoblastic differentiation markers including runt related transcription factor 2, osterix and alkaline phosphatase [27] compared to wild type cells, suggesting *Wnt5a* regulates bone formation. This is supported by histological analysis of long bones of *Wnt5a*^{-/-} mice, which exhibit significantly delayed chondrocyte hypertrophy and skeletal ossification compared to wild type mice [25]. *Wnt5a* induces its effects via several known receptors and co-receptors including Frizzled2 (FZD2), Frizzled5 (FZD5), and receptor tyrosine kinase-like orphan receptor 2 (ROR2), activating intracellular release of calcium, thereby activating PLC, PKC, CaMKII and calcineurin [28–32].

Although 1α,25(OH)₂D₃ and *Wnt5a* both regulate osteoblast and chondrocyte maturation and signal via calcium-dependent mechanisms, little is known about the role of components of the 1α,25(OH)₂D₃ membrane-associated receptor complex in *Wnt5a* calcium-dependent signaling. The purpose of this study was to determine if the same receptor complex and pathway signaling proteins that are critical for the 1α,25(OH)₂D₃ membrane-mediated pathway via Pdia3 are also important for *Wnt5a* calcium-dependent signaling. To address this question, we first verified *Wnt5a*-dependent activation of CaMKII, PLA₂, PKC, and PGE₂ release in MC3T3-E1 osteoblast-like cells and GC cells. Next, we determined the role of the 1α,25(OH)₂D₃ activated Pdia3-dependent membrane receptor complex and its downstream signaling proteins in *Wnt5a*-stimulated PKC activation using the MC3T3-E1 cell model. Finally, we determined the interactions between the 1α,25(OH)₂D₃ membrane-receptor complex and *Wnt5a* receptors with or without 1α,25(OH)₂D₃ or *Wnt5a* treatment in MC3T3-E1 cells.

2. Materials and methods

2.1. Reagents

Recombinant human/mouse *Wnt5a* was purchased from R&D Systems (Minneapolis, MN). 1α,25(OH)₂D₃ and PLAA were purchased

from Enzo Life Sciences (Plymouth Meeting, PA). The anti-PLAA polyclonal antibody was designed and developed by Strategic Diagnostics Inc. (Newark, DE) [12]. Rabbit antiserum against the N-terminal peptide of Pdia3 was purchased from Alpha Diagnostic International (San Antonio, TX) [33]. A polyclonal antibody to Cav-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ROR2 polyclonal antibody was from Cell Signaling Technology (Danvers, MA). Frizzled2 polyclonal antibody and Frizzled5 polyclonal antibody were from Abcam (San Francisco, CA). Monoclonal antibody to CaM was from Millipore (Billerica, MA). Myristoylated calmodulin kinase IINtide (mer-CaMKIINtide) peptide was from EMD Biosciences (Billerica, MA) and arachidonyl trifluoromethyl ketone (AACOCF₃) was from Abcam. All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless specified.

2.2. Cell culture

Wild type (WT) mouse MC3T3-E1 subclone 4 osteoblast-like cells (CRL-2593) were purchased from ATCC (Manassas, VA, USA). Stably silenced MC3T3-E1 cell lines for Pdia3 (shPdia3), PLAA (shPlaa), Cav-1 (shCav-1), VDR (shVdr), CaMKII-α (shCamk2a), and CaMKII-β (shCamk2b) were generated and characterized by our lab previously [9, 12,34]. Cells were plated at 10,000 cells/cm² and cultured in Minimum Essential Medium Alpha (α-MEM) (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone, Waltham, MA) and 1% penicillin–streptomycin (P/S) (Life Technologies). Osteoblastic differentiation of MC3T3-E1 cells was induced by culture in α-MEM supplemented with 10% FBS, 1% P/S and 50 μg/ml ascorbic acid 24 h after plating, and then every 48 h thereafter [35].

GC cells were isolated from costochondral cartilage of 100–125 g male Sprague–Dawley rats (Harlan, Indianapolis, IN). Rats were at the end of their adolescent growth spurt; therefore, their long bones were growing at a reduced rate. The culture system used in this study was described previously in detail [36,37]. Briefly, fourth passage cultures of GC chondrocytes were plated at 10,000 cells/cm² and cultured in Dulbecco's modified Eagle's medium (Mediatech) containing 10% FBS, 1% P/S, and 50 μg/ml ascorbic acid.

All cells were cultured at 37 °C with 5% CO₂ and 100% humidity. Confluent cultures were treated for experiments as described below. The approximate density of MC3T3-E1 osteoblast-like cells at the time of treatment was 80,000 cells/cm².

2.3. Time course and dose–response of PKC activity to *Wnt5a*

To assess the dose-dependent effects of *Wnt5a* on PKC, GC cells were treated for 9 min and MC3T3-E1 cells were treated for 15 min with 50, 125 and 200 ng/ml *Wnt5a* [26,38], time points previously demonstrated to be optimal for activation of PKC by 1α,25(OH)₂D₃ in these cell types [12,39]. After treatment, cell layers were washed with phosphate buffered saline (PBS) and then were lysed in RIPA buffer (20 mM Tris–HCl, 150 mM NaCl, 5 mM disodium EDTA, 1% Nonidet P-40). PKC activity was measured in cell layer lysates using a commercially available kit following manufacturer's instructions (GE Healthcare, Piscataway, NJ) and data were normalized to total protein (Pierce BCA Protein Assay, Thermo Fisher Scientific, Waltham, MA).

Next, the time course of PKC activation by *Wnt5a* was examined. GC cells and MC3T3-E1 cells were treated with 125 ng/ml [26,38] *Wnt5a* for 6, 9, 15, or 30 min. PKC activity was measured as described above.

2.4. Time course of *Wnt5a* effect on CaMKII activity

GC and MC3T3-E1 cells were treated for 6, 9, 15, and 30 min with 125 ng/ml *Wnt5a* and cell layer lysates were assayed for CaMKII activity. CaMKII activity was measured using a commercially available assay following manufacturer's instructions (SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System, Promega, Madison, WI). CaMKII activity was normalized to total protein in the cell lysate.

2.5. Time course of Wnt5a effect on PLA₂ activity

GC and MC3T3-E1 cells were treated for 6, 9, 15, or 30 min with 125 ng/ml Wnt5a. After washing the cell layers with PBS, the cell layers were lysed and assayed for PLA₂ activity using a commercially available kit (cPLA₂ Assay kit, 765021, Cayman Chemical, Ann Arbor, MI). PLA₂ data were normalized to total protein.

2.6. Time course of Wnt5a effect on PGE₂ release

GC and MC3T3-E1 cells were treated for 6, 9, 15, and 30 min with 125 ng/ml Wnt5a. At the end of incubation, conditioned media were acidified and PGE₂ was measured using a commercially available kit (Prostaglandin E₂ [¹²⁵I]-RIA kit, Perkin Elmer, Waltham, MA). PGE₂ levels were normalized to total DNA (Quant-iTTM PicoGreen® dsDNA Assay kit, Life Technologies).

2.7. Role of vitamin D signaling components in Wnt5a-induced PKC activity

Based on the results of time-course and dose-response studies, the treatment conditions resulting in the highest PKC activity (125 ng/ml Wnt5a for 9 min in GC cells and 125 ng/ml Wnt5a for 15 min in MC3T3-E1 osteoblast-like cells) were selected for subsequent experiments unless specified in the text.

To determine the effects of Pdia3, Vdr, and Cav1 silencing on Wnt5a-induced PKC activity, wild type (WT), shPdia3, shVdr, and shCav-1 cells were treated with Wnt5a and PKC measured. These results were confirmed using Pdia3 and VDR blocking antibodies in GC chondrocytes and MC3T3-E1 cells. Cells were pretreated with either anti-Pdia3 or anti-VDR antibodies for 30 min, and PKC activity measured after Wnt5a treatment.

Methyl-beta-cyclodextrin (β-CD) disrupts lipids rafts and caveolae by binding cholesterol and removing it from the plasma membranes. To determine the effects of caveolae destruction on PKC activation, MC3T3-E1 cells were treated with 10 mM of β-CD for 30 min in serum-free media, as described previously [40]. At the end of incubation, cell layers were rinsed with serum free media. Cells were then treated with Wnt5a and cell layer lysates were assayed for PKC activity.

2.8. Role of calmodulin in Wnt5a-induced PKC activity

MC3T3-E1 cells were pre-treated with 0.1, 1, or 10 μM calmodulin inhibitor W-7 for 30 min [41], then cells were treated with Wnt5a for 15 min and cell layer lysates were assayed for PKC activity. To investigate the effect of CaMKII inhibition on 1α,25(OH)₂D₃-dependent PKC activation, MC3T3-E1 cells were treated for 30 min with 1.25, 2.5, and 5 μM CaMKII peptide-inhibitor mer-CaMKIINtide [42] followed by Wnt5a. Cell layer lysates were assayed for PKC activity. To investigate the effects of Camk2a and Camk2b silencing on Wnt5a-stimulated PKC activation, confluent WT, shCamk2a, and shCamk2a MC3T3-E1 cells were treated with Wnt5a. Cell layer lysates were assayed for PKC.

2.9. Role of PLAA in Wnt5a-induced PKC activity

Cells were pretreated with anti-PLAA antibody (PLAA Ab) for 30 min followed by Wnt5a for 15 min. Cell layer lysates were assayed for PKC activity. To confirm these findings, PKC activity was measured in WT and shPlaa MC3T3-E1 cells after Wnt5a treatment. To investigate the effects of PLA₂ inhibition on PKC activity, MC3T3-E1 osteoblast-like cells were pretreated with 0.1, 1, and 10 μM AACOCF₃ for 30 min followed by Wnt5a. PKC activity was measured in cell layer lysates. AACOCF₃ has been shown in other studies to inhibit PLA₂ in these cells [43].

2.10. Effects of Wnt5a and 1α,25(OH)₂D₃ co-treatment on regulation of PKC activity

MC3T3-E1 cells were treated with 0, 10⁻¹⁰, 10⁻⁹, or 10⁻⁸ M 1α,25(OH)₂D₃. Wnt5a was added to one-half of the cultures at the concentration of 125 ng/ml. At the end of the 15-min incubation, cell layer lysates were assayed for PKC. To assess the effects of Wnt5a in 1α,25(OH)₂D₃ regulation of PKC activity, MC3T3-E1 cells were treated with 10⁻⁸ M 1α,25(OH)₂D₃ and 0, 50, 87.5 or 125 ng/ml Wnt5a for 15 min. At the end of the incubation, the cell layer lysates were assayed for PKC.

2.11. Roles of ROR2, FZD2 and FZD5 in 1α,25(OH)₂D₃-induced PKC activity

MC3T3-E1 cells were pretreated with anti-Frizzled2 (FZD2 Ab), anti-Frizzled5 (FZD5 Ab), or anti-ROR2 (ROR2 Ab) antibodies for 30 min, and were then treated with 10⁻⁸ M 1α,25(OH)₂D₃ for 15 min. PKC activity was measured in cell layer lysates.

2.12. Role of ROR2 in 1α,25(OH)₂D₃-induced CaMKII activity

MC3T3-E1 cells were pretreated with anti-ROR2 antibodies for 30 min, and were then treated with 10⁻⁸ M 1α,25(OH)₂D₃ for 15 min. CaMKII activity was measured in cell layer lysates.

2.13. Role of ROR2 on PLAA-induced CaMKII activity

MC3T3-E1 osteoblasts were pretreated with anti-ROR2 antibodies for 30 min, and were then treated with 10⁻⁶ M PLAA peptide for 15 min. CaMKII activity was measured in cell layer lysates.

2.14. Caveolae isolation

Pdia3 exists in plasma membrane caveolae of osteoblasts [9]. To determine the plasma membrane localization of FZD2, FZD5 and ROR2, plasma membranes and caveolae were isolated using a detergent-free method as described previously [44]. Briefly, confluent MC3T3-E1 cultures were harvested by scraping while in isolation buffer (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8) and were homogenized using a tissue grinder (20 strokes; 10 strokes clockwise and 10 strokes counter-clockwise). Homogenates were centrifuged at 20,000g for 10 min. The supernatant was collected and layered on top of isolation buffer containing 30% Percoll® (GE Healthcare, Piscataway, NJ). The pellet, including nucleus, mitochondria, and endoplasmic reticulum, was discarded. Samples were centrifuged at 84,000g for 30 min. Syringe needles (18G) were used to collect the plasma membrane fraction from the gradient column. The isolated fraction was layered over a 10%–20% OptiPrep gradient (Sigma Aldrich, St. Louis, MO), then centrifuged at 52,000g for another 4 h. Plasma membrane sub-fractions were collected from the tube, resulting in isolation of thirteen fractions. Caveolae were observed as an opaque band that was collected in fraction 3.

2.15. Western blots

Whole cell layer lysates and plasma membrane fractions (50 μg protein) were loaded onto 4%–20% Mini-PROTEAN® TGXTM precast polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred to low-fluorescence PVDF membranes (Bio-Rad) using a Trans-Blot® TurboTM Transfer System (Bio-Rad). Membranes were incubated with blocking buffer (LI-COR, Lincoln, NE) for 1 h. Subsequently, the membranes were incubated with antibodies against Cav-1, FZD2, FZD5, and ROR2 overnight. The next day, the membranes were incubated for 1 h with IRDye 800CW conjugated goat anti-rabbit IgG secondary antibodies (LI-COR) in blocking buffer containing 0.2% Tween-20, and 0.01% SDS. Following three washes with PBS containing 0.05%

Tween-20 membranes were imaged using the LI-COR Odyssey® CLx Infrared Imaging System.

2.16. Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and Wnt5a on receptor complex interactions

MC3T3-E1 cells were treated with 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ or its vehicle (ethanol), or 125 ng/ml Wnt5a or its vehicle (cell culture medium) for 15 min. At the end of treatment, cell layers were washed with PBS and cell layers were lysed in RIPA buffer containing 100 mM sodium fluoride, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride. Protein samples (500 μg) were pre-cleared by incubation in 5 μg of rabbit IgG conjugated to Dynabeads® Protein A (Life Technologies) at 4 °C for 1 h. The beads were separated from solution using a magnet. To immunoprecipitate Pdia3, PLAA, Cav-1 and CaM protein complexes, anti-Pdia3, anti-PLAA, anti-Cav-1, and anti-CaM antibodies were covalently coupled to the Dynabeads Protein A according to the manufacturer's protocol. Pre-cleared protein samples were mixed with antibody coated Dynabeads and incubated at 4 °C overnight with continuous agitation. Dynabeads were recovered using a magnet and were washed three times with 0.05% Tween-20 in PBS. Precipitated proteins were eluted in elution buffer, then diluted in Tris–glycine SDS sample loading buffer (Bio-Rad) and boiled for 5 min. Immunoprecipitated samples were examined by Western blot (ONE-HOUR Western™ Fluorescent Kit, Genscript, Piscataway, NJ).

2.17. Statistical analysis

For each experiment, data points represent the mean \pm standard error of the mean (SEM) of six individual cultures, per variable. Each experiment was repeated at least three times to ensure validity of the data. Statistical significance was assessed by analysis of variance and post hoc testing performed using Bonferroni's modification of Student's *t*-test for multiple comparisons (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). *P*-values <0.05 were considered significant.

3. Results

3.1. Wnt5a rapidly activates PKC, CaMKII, and PLA_2 and triggers PGE_2 release

Wnt5a regulated the activity of PKC in GC and MC3T3-E1 cells in a dose- and time-dependent manner. Similarly, the effects of Wnt5a on CaMKII and PLA_2 activities and PGE_2 release were time-dependent and rapid. Wnt5a protein increased PKC-specific activity in a dose-dependent manner in GC cells with the highest stimulatory effects of the peptide observed at 125 ng/ml concentration (Fig. 1A). Wnt5a activated PKC in a dose-dependent manner in MC3T3-E1 cells, with the highest stimulatory effect observed at 125 ng/ml (Fig. 1B). Wnt5a activated PKC in GC cells within 9 min of treatment, and PKC remained significantly higher than control at 15 and 30 min after treatment (Fig. 1C).

We further assessed whether Wnt5a acts on cells by a mechanism similar to that used by $1\alpha,25(\text{OH})_2\text{D}_3$. Wnt5a increased CaMKII activity at 9 and 15 min after treatment in GC chondrocytes (Fig. 1D). Wnt5a increased PLA_2 activity (Fig. 1E) and PGE_2 release (Fig. 1F) in GC cells at both 9 min and 30 min after treatment. Similarly, Wnt5a caused a rapid increase in PKC activity in MC3T3-E1 cells at 9 min that remained elevated until 30 min, with a peak at 15 min (Fig. 1G). Wnt5a increased CaMKII activity at 6, 9, and 30 min in MC3T3-E1 osteoblast-like cells (Fig. 1H). Similarly, the effect of Wnt5a on PLA_2 activity (Fig. 1I) and PGE_2 release (Fig. 1J) was time-dependent and significant increases were observed at 9 and 30 min after the treatment.

3.2. Pdia3, VDR, and lipid rafts, but not Cav-1, are required for the PKC activation in response to Wnt5a

Pdia3, VDR, and plasma membrane lipid raft structures were involved in Wnt5a-stimulated rapid activation of PKC in MC3T3-E1 osteoblasts, while Cav-1 was not required. Unlike WT cells, shPdia3 osteoblasts did not increase in PKC activity in response to Wnt5a (Fig. 2A). Wnt5a stimulated PKC activity in shVdr cells was significantly lower than in WT cells (Fig. 2B). However, Wnt5a failed to increase PKC in shCav-1 osteoblasts (Fig. 2C). Likewise, Pdia3 antibody (Fig. 2D) and VDR antibody (Fig. 2E) significantly reduced the Wnt5a-induced increase in PKC activity in MC3T3-E1 cells. Although Wnt5a stimulated PKC activity in cells pretreated with lipid raft disruptor β -CD, the increase was significantly lower when compared to the β -CD untreated group (Fig. 2F).

3.3. CaM, CaMKII- α , PLAA, and PLA_2 are required for the rapid activation of PKC in response to Wnt5a

In MC3T3-E1 cells, Wnt5a-stimulated activation of PKC depends on upstream actions of CaM, CaMKII- α , PLAA, and PLA_2 . Calmodulin inhibitor W-7 blocked activation of PKC in response to Wnt5a at 1 and 10 μM , but not at 0.1 μM (Fig. 3A). mer-CaMKIINtide inhibited the Wnt5a induced increase in PKC activity in a comparable manner to W-7. mer-CaMKIINtide significantly reduced stimulatory effects of Wnt5a on PKC activation at 1.25 μM , and completely blocked the effect at 2.5 and 5 μM (Fig. 3B). Wnt5a significantly increased PKC activity in WT and shCamk2b MC3T3-E1 cells, but this effect was prevented in shCamk2a cells (Fig. 3C). PLAA antibody significantly reduced the Wnt5a-induced increase in PKC activity in MC3T3-E1 cells, whereas IgG had no effect (Fig. 3D). Similarly, Wnt5a increased PKC activity in MC3T3-E1 cells, and effect was blocked in shPlaa cells (Fig. 3E). PLA_2 inhibitor AACOCF3 significantly reduced stimulatory effects of Wnt5a on PKC activation at 0.1 μM , and completely blocked the effect at 1 and 10 μM (Fig. 3F).

3.4. Co-treatment with Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ reduces PKC activation in a dose-dependent manner

Given that both $1\alpha,25(\text{OH})_2\text{D}_3$ and Wnt5a promote maturation of osteoblasts and use Pdia3 to induce their effects, we tested the effects of their co-treatment on the induction of their signaling pathways in MC3T3-E1 osteoblast-like cells, specifically examining PKC activation. In MC3T3-E1 cells, Wnt5a caused a 75% increase in PKC activity (Fig. 4A). Addition of $1\alpha,25(\text{OH})_2\text{D}_3$ reduced the stimulatory effect of Wnt5a in a dose-dependent manner. At the highest concentration of the hormone, PKC was increased by 30% in control cultures but the effect of Wnt5a was abolished. The effect of Wnt5a on PKC in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ also depended on Wnt5a concentration. Co-treatment with 50 ng/ml Wnt5a caused a 2-fold increase in $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated PKC activity compared to cultures treated with only $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig. 4B). However, as the concentration of Wnt5a increased, $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated PKC activation was suppressed.

3.5. ROR2, FZD2, and FZD5 are localized in caveolae and ROR2 is crucial for $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated activation of PKC and CaMKII

Given, the key roles ROR2, FZD2 and FZD5 play in Wnt5a signaling, we assessed their role on $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated rapid activation of PKC and CaMKII. ROR2, but not FZD2 and FZD5 mediated the $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated rapid activation of PKC and CaMKII in MC3T3-E1 osteoblasts-like cells. Anti-ROR2 antibody abolished the $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent increase in PKC activity in MC3T3-E1 cells (Fig. 5A), whereas, IgG had no effect. Conversely, blocking FZD2 and FZD5 receptors had no effect on activation of PKC in response to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. Similarly, anti-ROR2 antibody abolished the

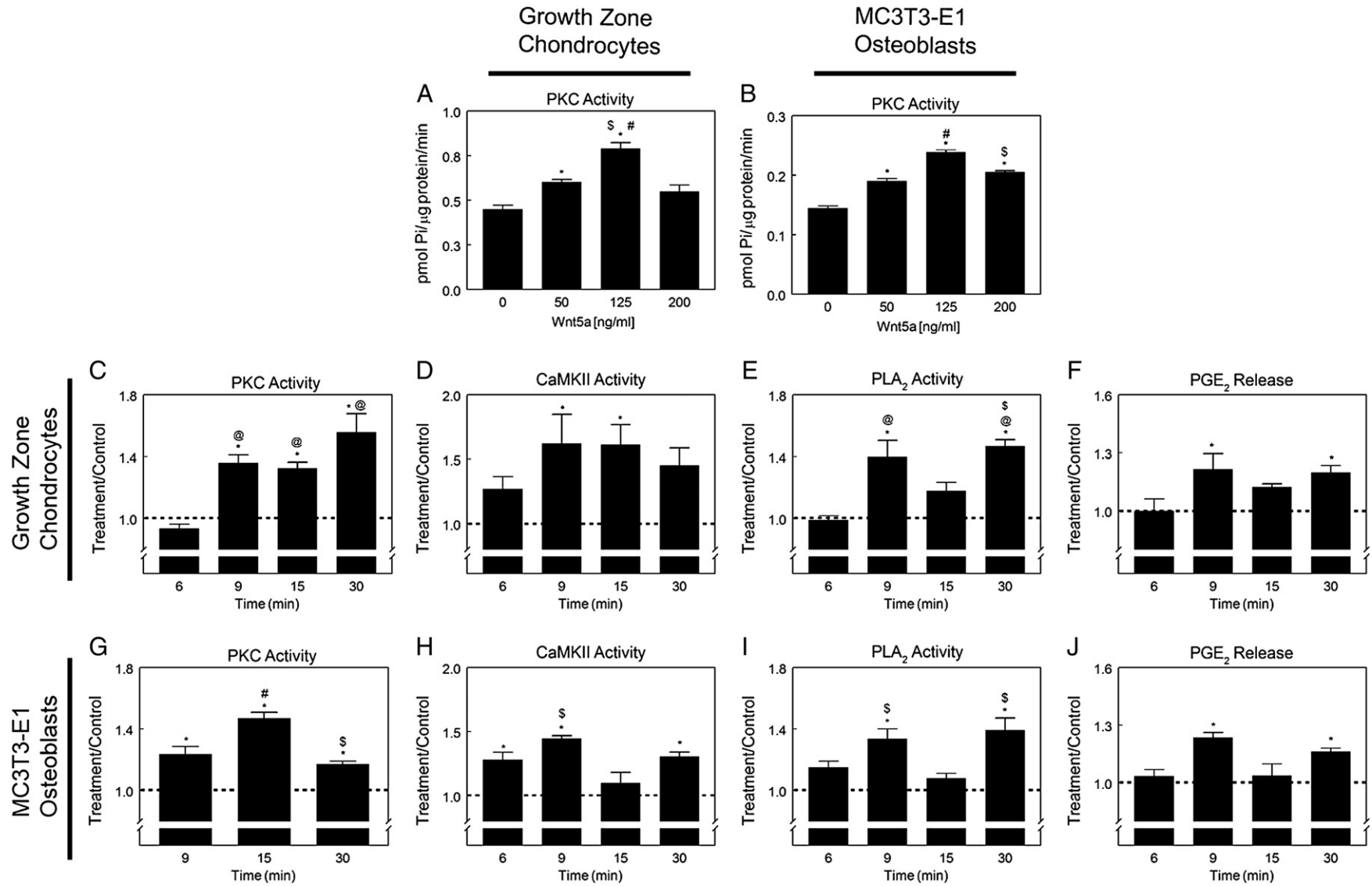


Fig. 1. Effects of Wnt5a treatment on PKC, CaMKII, and PLA₂ activation, and PGE₂ release and growth zone chondrocytes (GC) (A) and MC3T3-E1 osteoblast-like cells (B). **p* < 0.05, versus 0 ng/ml Wnt5a; #*p* < 0.05, versus 50 ng/ml Wnt5a; \$*p* < 0.05, versus 50 ng/ml Wnt5a. Time-course of Wnt5a-induced PKC (C, G), CaMKII (D, H) and PLA₂ (E, I) activation in GC (C–F) and MC3T3-E1 (G–J) cells. Treatment over control ratios were calculated for each parameter. The dashed line represents the value for the control cultures, which was set to 1. **p* < 0.05, 1 α ,25(OH)₂D₃ treatment versus control; @*p* < 0.05, versus 6 min; #*p* < 0.05, versus 9 min; \$*p* < 0.05, versus 6 min.

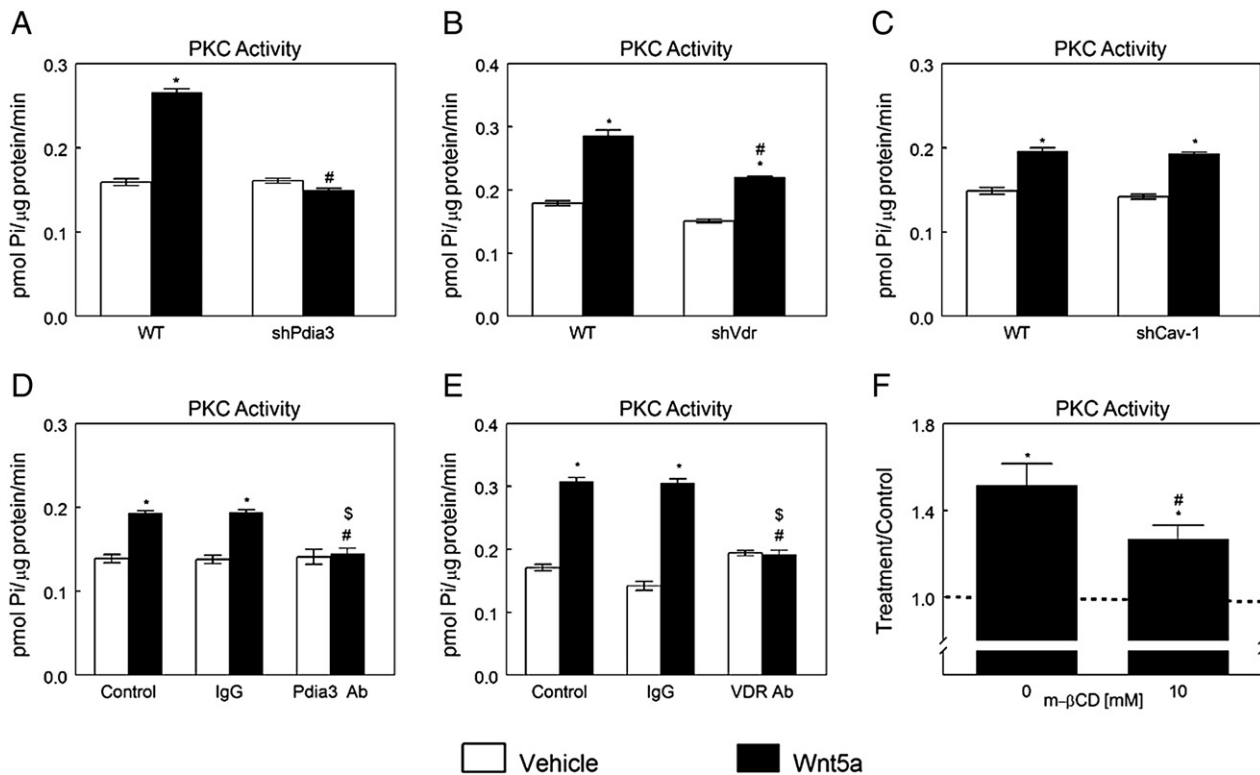


Fig. 2. Effects of Pdia3, Vdr and Cav-1 silencing or blocking and β -CD on Wnt5a-stimulated activation of PKC in MC3T3-E1 cells. Wnt5a-induced PKC activation in wild-type (WT), shPdia3 (A), shVdr (B) and shCav-1 (C) MC3T3-E1 cells. * $p < 0.05$, treatment versus control; # $p < 0.05$, versus Wnt5a treated WT. Effect of Pdia3 blocking (D) or VDR blocking (E) on Wnt5a-induced PKC activation. * $p < 0.05$, treatment versus control; # $p < 0.05$, versus Wnt5a treated control group; \$ $p < 0.05$, versus Wnt5a treated IgG group. (F) Effect of β -CD on Wnt5a-induced PKC activation. Treatment over control ratios were calculated for each parameter. The dashed line represents the value for the control cultures, which was set to 1. * $p < 0.05$, treatment versus control.

$1\alpha,25(\text{OH})_2\text{D}_3$ -dependent increase in CaMKII activity in MC3T3-E1 cells (Fig. 5B). Furthermore, anti-ROR2 antibody had no effect on PLAA-induced CaMKII activation (Fig. 5C). Western blots of the plasma membrane fractions of MC3T3-E1 cells indicated that ROR2, FZD2, and FZD5 were present in the plasma membranes with their greatest concentration in fraction 3, which represents caveolae microdomains (Fig. 5D).

3.6. $1\alpha,25(\text{OH})_2\text{D}_3$ alters the interactions between $1\alpha,25(\text{OH})_2\text{D}_3$ receptor complex and Wnt5a receptors

Immunoprecipitation studies confirmed the interaction between components of $1\alpha,25(\text{OH})_2\text{D}_3$ receptor complex and Wnt5a receptors. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment altered some of these interactions. Samples of MC3T3-E1 whole cell lysates immunoprecipitated using antibodies to Pdia3 (IP:Pdia3) were positive for FZD2, FZD5, and ROR2 (Fig. 6A). Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ for 15 min had no effect on Pdia3:FRZ5 but increased Pdia3:FZD2 and Pdia3:ROR2. Western blots of whole cell lysates immunoprecipitated with antibodies to PLAA (IP:PLAA) demonstrated an increase in FZD2-associated PLAA after addition of $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig. 6B). FZD5 and ROR2 interacted with PLAA, but $1\alpha,25(\text{OH})_2\text{D}_3$ treatment had no effect on these interactions. Cav-1 (IP:Cav-1) interacted with FZD2, FZD5, and ROR2 (Fig. 6C). Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ for 15 min had no effect on FZD2 and FZD5 but reduced ROR2. Immunoprecipitation of CaM (IP:CaM) demonstrated interaction with FZD5 with or without $1\alpha,25(\text{OH})_2\text{D}_3$ treatment (Fig. 6D). FZD2 and ROR2 also interacted with CaM and $1\alpha,25(\text{OH})_2\text{D}_3$ treatment reduced their interactions with CaM.

3.7. Wnt5a alters the interactions between $1\alpha,25(\text{OH})_2\text{D}_3$ receptor complex and Wnt5a receptors

Immunoprecipitates of MC3T3-E1 whole cell lysates using antibodies to Pdia3 (IP:Pdia3) were positive for FZD2 and FZD5 (Fig. 7A). Treatment with Wnt5a for 15 min had no effect on FZD2's interaction with Pdia3 but increased FZD5. Western blots of whole cell lysates immunoprecipitated with antibodies to PLAA (IP:PLAA) demonstrated interaction with FZD2 and FZD5 with and without $1\alpha,25(\text{OH})_2\text{D}_3$ treatment (Fig. 7B). ROR2 also interacted with PLAA, and Wnt5a treatment reduced its interaction with PLAA. Immunoprecipitation of Cav-1 (IP:Cav-1) demonstrated interactions with FZD2, FZD5 and ROR2 (Fig. 7C). While Wnt5a treatment reduced FZD2 and ROR2 interactions with Cav-1, it had no effect on FZD5 interactions with Cav-1. Immunoprecipitation of CaM (IP:CaM) demonstrated interaction with FRZD5 with or without Wnt5a treatment (Fig. 7D). FZD2 and ROR2 also interacted with CaM. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment increased FZD2 and CaM interaction and decreased ROR2 and CaM interaction.

4. Conclusions

This study demonstrates that signaling proteins critical for the $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated pathway are also crucial for Wnt5a calcium-dependent signaling. Events at the signaling level indicated that Wnt5a calcium-dependent signaling works through a mechanism involving the CaMKII/PLA₂/PGE₂/PKC pathway. Moreover, silencing or blocking of Pdia3, PLAA and VDR and inhibition of CaM, CaMKII and PLA₂ affected the activity of PKC in response to Wnt5a treatment. In contrast, PKC activity was unaffected in Cav-1 silenced cells,

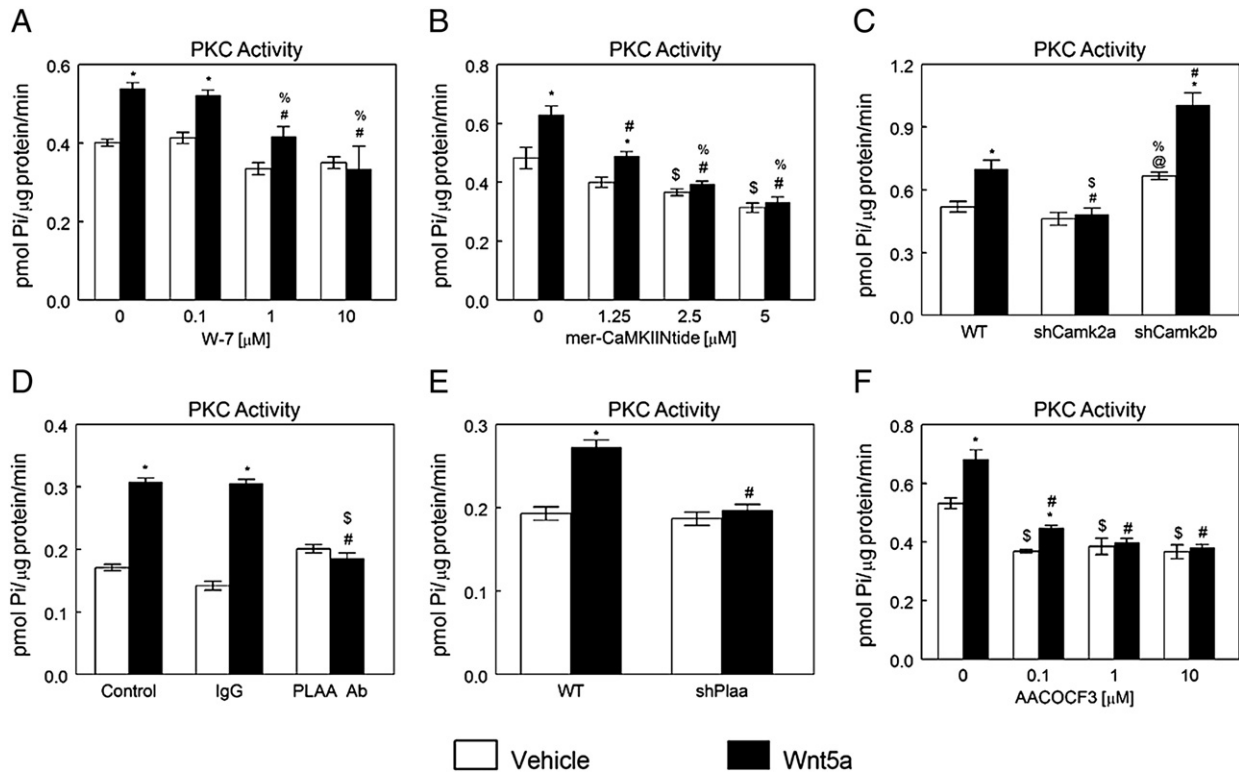


Fig. 3. Effects of CaM, CaMKII, PLA₂ and PLA₂ inhibition and Plaa, Camk2a and Camk2b silencing on Wnt5a-stimulated activation of PKC in MC3T3-E1 cells. (A) Effect of CaM inhibition on Wnt5a-induced PKC activation. **p* < 0.05, treatment versus control; #*p* < 0.05, versus 0 μM W-7 + Wnt5a; \$*p* < 0.05, versus 0.1 μM W-7 + Wnt5a. (B) Effect of CaMKII inhibition on Wnt5a-induced PKC activation. **p* < 0.05, treatment versus control; #*p* < 0.05, versus 0 μM mer-CaMKIINtide + Wnt5a; \$*p* < 0.05, versus 0.1 μM mer-CaMKIINtide + vehicle; %*p* < 0.05, versus 1.25 μM mer-CaMKIINtide + Wnt5a. (C) Effect of Wnt5a treatment on PKC activation in wild-type (WT), shCamk2a, and shCamk2b MC3T3-E1 cells. **p* < 0.05, treatment versus control; #*p* < 0.05, versus Wnt5a-treated WT; \$*p* < 0.05, versus Wnt5a treated shCamk2b; @*p* < 0.05, versus vehicle treated WT; **p* < 0.05, versus vehicle treated shCamk2a. (D) Effect on PLA₂ blocking on Wnt5a-induced PKC activation. **p* < 0.05, treatment versus control; #*p* < 0.05, versus Wnt5a treated control group; \$*p* < 0.05, versus Wnt5a treated IgG group. (E) Effect of Plaa silencing on Wnt5-stimulated PKC activation. **p* < 0.05, treatment versus control; #*p* < 0.05, versus Wnt5a-treated WT cells. (F) Effect of PLA₂ inhibitor, AACOCF₃, on Wnt5a-induced PKC activation. **p* < 0.05, treatment versus control; #*p* < 0.05, versus 0 μM AACOCF₃ + Wnt5a; \$*p* < 0.05, 0 μM AACOCF₃ + vehicle versus 0.1 μM AACOCF₃ + Wnt5a.

but the stimulatory effect of Wnt5a was decreased in cells treated with β-CD. Furthermore, Western blots of plasma membrane fractions indicated that ROR2, FZD2, and FZD5 are localized in caveolae fraction. Blocking of ROR2 abolished the stimulatory effects of 1α,25(OH)₂D₃ on PKC and CaMKII activations. This study also provides mechanistic information by showing that the 1α,25(OH)₂D₃ receptor complex and its downstream mediators form complexes with Wnt5a receptors, and

these complexes respond to both 1α,25(OH)₂D₃ and Wnt5a treatment by altering some of their protein–protein interactions.

Similar to 1α,25(OH)₂D₃ signaling, Wnt5a time course studies indicate a rapid increase in CaMKII, PLA₂ and PKC activities and PGE₂ release in GC chondrocytes and MC3T3-E1 osteoblast-like cells. While the profiles of time points at which Wnt5a activated PLA₂ and triggered PGE₂ release were similar between GC and MC3T3-E1 cells, differences

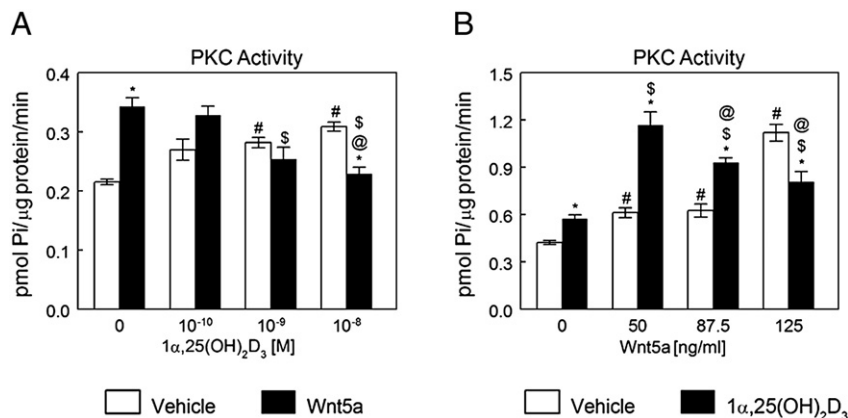


Fig. 4. Effect of 1α,25(OH)₂D₃ on Wnt5a-dependent PKC activation and effect of Wnt5a in 1α,25(OH)₂D₃ regulation of PKC activity. (A) MC3T3-E1 cells were treated for 15 min with 10^{−10}, 10^{−9} and 10^{−8} M 1α,25(OH)₂D₃. Wnt5a was added to one-half of the cultures at the concentration of 125 ng/ml, at the time of the treatment. At the end of incubation, cell layers were collected for PKC assay. **p* < 0.05, treatment versus control; #*p* < 0.05, versus 0 M 1α,25(OH)₂D₃ + vehicle; \$*p* < 0.05, versus 0 M 1α,25(OH)₂D₃ + Wnt5a; @*p* < 0.05 versus 10^{−10} M 1α,25(OH)₂D₃ + Wnt5a. (B) MC3T3-E1 cells were treated with 10^{−8} M 1α,25(OH)₂D₃ in the presence and absence of 50, 87.5 and 125 ng/ml Wnt5a. At the end of the incubation, the cell layers were collected for PKC assay. **p* < 0.05, treatment versus control; #*p* < 0.05, versus 0 ng/ml Wnt5a + vehicle; \$*p* < 0.05, versus 1α,25(OH)₂D₃ + 0 ng/ml Wnt5a; @*p* < 0.05 versus 1α,25(OH)₂D₃ + 5 ng/ml Wnt5a.

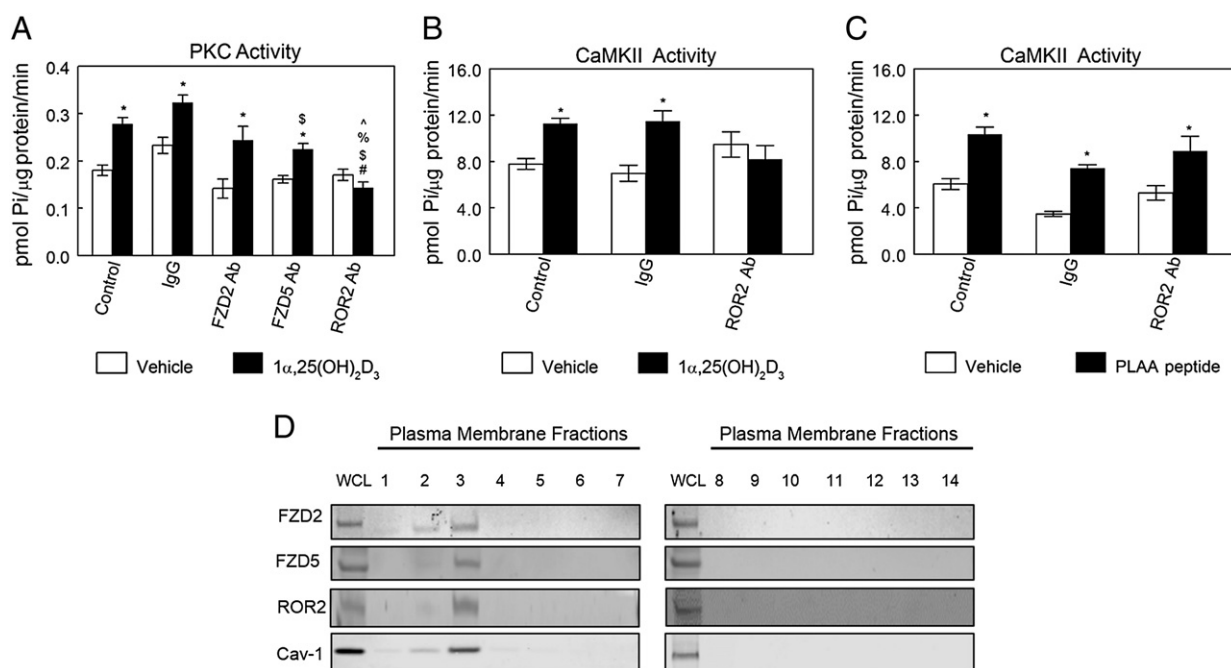


Fig. 5. Roles of FZD2, FZD5 and ROR2 in $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent PKC activation and their subcellular localization. (A) Effects of FZD2, FZD5 or ROR2 blocking on $1\alpha,25(\text{OH})_2\text{D}_3$ -induced PKC activation. * $p < 0.05$, treatment versus control; $^{\#}p < 0.05$, versus vehicle treated Control group; $^{\$}p < 0.05$, versus IgG + $1\alpha,25(\text{OH})_2\text{D}_3$; $^{\wedge}p < 0.05$ versus ROR2 Ab + $1\alpha,25(\text{OH})_2\text{D}_3$. (B) Effect of ROR2 blocking on $1\alpha,25(\text{OH})_2\text{D}_3$ -induced CaMKII activation. * $p < 0.05$, treatment versus control. (C) Effect of ROR2 blocking on PLAA peptide-stimulated CaMKII activation. * $p < 0.05$, treatment versus control. (D) Subcellular localization of FZD2, FZD5 and ROR2. Plasma membrane fractions were isolated as described. Presence of FZD2, FZD5, ROR2 and Cav-1 in fractions was examined by Western blot. Each figure is a representative experiment repeated three times with similar results.

were observed in PKC and CaMKII activities. One reason that may contribute to such a difference is that while GC cells are primary cartilage cells isolated from the rat costochondral cartilage growth zone, MC3T3-E1 osteoblast-like cells are a cell line derived from mouse calvaria. The second potential reason for such an observation is that growth zone chondrocytes used in these experiments were isolated from 100–125 g male Sprague–Dawley rats, and these rats were at the end of their adolescent growth spurt while the mouse osteoblast-like MC3T3-E1 cell line was derived from a newborn mouse.

When GC and MC3T3-E1 cells were treated with Wnt5a, the increase in PLA₂ activity and PGE₂ release occurred in a cyclic manner. A rapid elevation was observed at 9 min after the treatment, which was followed by a decline at 15 min and a second increase at 30 min post Wnt5a treatment. The initial increase can be attributed to a rapid Wnt5a-induced use of available factors required for activation of PLA₂ and downstream PGE₂ release, as well as an increase in intracellular calcium and activation of CaMKII [13,45,46]. The increase observed at 30 min

might be the consequence of cross-talk with another pathway that involves in activation of PLA₂ or it could result from production of new factors triggered by Wnt5a action. Previously, we reported that PLAA, VDR, Cav-1 and caveolae are involved in $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated signaling by Pdia3 [9,12,40,47]. VDR has also been shown by others to mediate rapid effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on Ca^{2+} transport via voltage gated Ca^{2+} channels [48]. Similar to $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated signaling, our results indicated that Pdia3, PLAA, and VDR are critical for Wnt5a calcium-dependent pathway. However, to our surprise, silenced Cav-1 osteoblasts activated PKC in response to Wnt5a treatment, suggesting that Cav-1 is not necessary for the Wnt5a calcium-dependent pathway. To further investigate the role of lipid rafts in Wnt5a calcium-dependent pathway, we subjected the cells to β -CD. PKC activity increased in cells pretreated with β -CD but the increase was significantly lower than the β -CD-untreated group. Collectively, these studies indicate that Wnt5a induces its effects partially via lipid rafts and caveolae are not required to mediate Wnt5a effects. Previously, FZD5 and FZD2 proteins

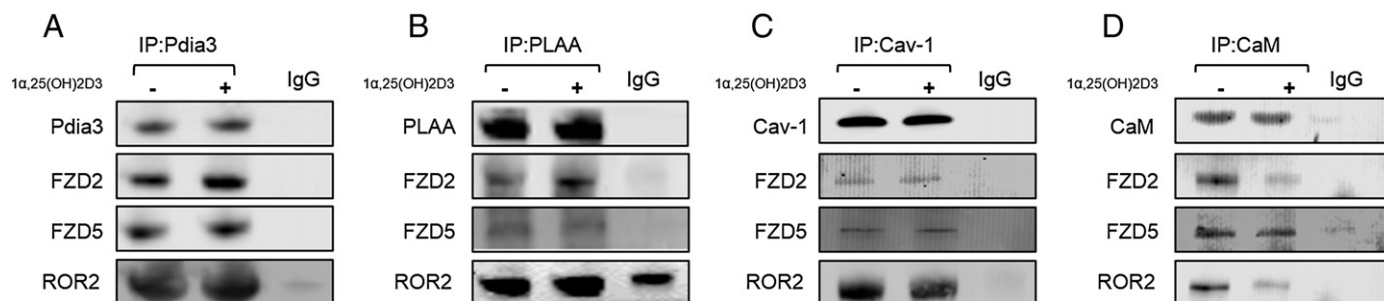


Fig. 6. Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ treatment on interactions between $1\alpha,25(\text{OH})_2\text{D}_3$ receptor complex and Wnt5a receptors. MC3T3-E1 cells were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ for 15 min. Whole cell lysates were isolated as described. (A) Pdia3 was immunoprecipitated and subjected to Western blot. The membranes were incubated with Ab Pdia3, Ab FZD2, anti-FZD5, and anti-ROR2 antibodies. (B) PLAA was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-PLAA, anti-FZD2, anti-FZD5, and anti-ROR2 antibodies. (C) Cav-1 was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-Cav-1, anti-FZD2, anti-FZD5, and anti-ROR2 antibodies. (D) CaM was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-CaM, anti-FZD2, anti-FZD5, and anti-ROR2 antibodies. Each figure is a representative experiment repeated three times with similar results.

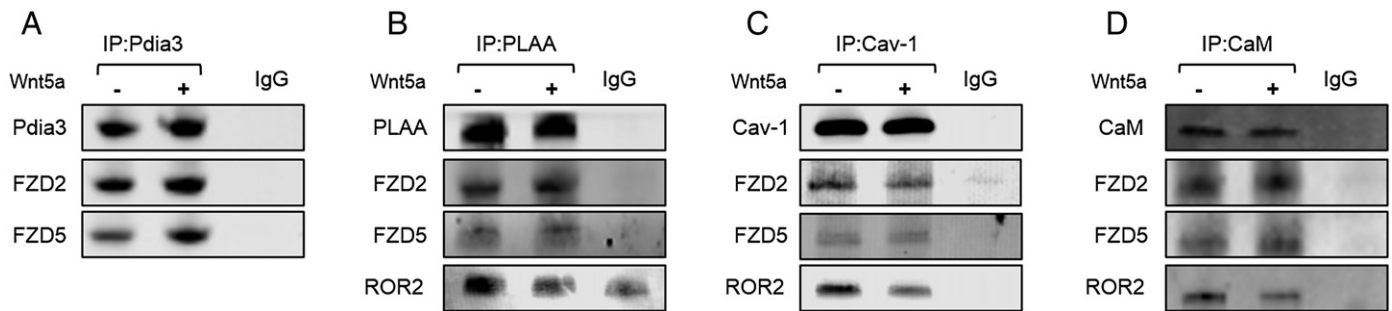


Fig. 7. Effect of Wnt5a treatment on interactions between $1\alpha,25(\text{OH})_2\text{D}_3$ receptor complex and Wnt5a receptors. MC3T3-E1 cells were treated with Wnt5a for 15 min. Whole cell lysates were isolated as described. (A) Pdia3 was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-Pdia3, anti-FZD2 and anti-FZD5 antibodies. (B) PLAA was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-PLAA, anti-FZD2, anti-FZD5 and anti-ROR2 antibodies. (C) Cav-1 was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-Cav-1, anti-FZD2, anti-FZD5 and anti-ROR2 antibodies. (D) CaM was immunoprecipitated and subjected to Western blot. The membranes were incubated with the anti-CaM, anti-FZD2, anti-FZD5 and anti-ROR2 antibodies. Each figure is a representative experiment repeated three times with similar results.

have been reported to be found in clathrin coated pits, suggesting that Wnt5a regulates its pathway via this group of lipid rafts [49].

Our inhibitor study indicates that CaM, CaMKII, and PLA_2 are critical for Wnt5a stimulated PKC activation. We previously reported that CaM plays a critical role in $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated pathway and its inhibition blocks $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated rapid activation of PKC [13]. In the present study, we found that CaM inhibition by W-7 abolished Wnt5a stimulated activation of PKC in a dose-dependent manner. Our hypothesis that CaM is required for Wnt5a stimulated activation of PKC is also supported by the observation that CaM inhibition suppresses PKC translocation in response to phorbol 12-myristate 13-acetate (PMA) treatment in the rat aorta [50]. In our previous work, we reported that CaMKII, isoform α , is necessary for $1\alpha,25(\text{OH})_2\text{D}_3$ membrane stimulated activation of PLA_2 , PKC and PGE_2 release [13]. Using mer-CaMKIINtide to inhibit the effect of CaMKII, we tested the role of CaMKII in Wnt5a induced PKC activation. In the current study, we found that CaMKII inhibition abolished Wnt5a stimulated activation of PKC in a dose-dependent manner. Our hypothesis that CaMKII is required for Wnt5a induced activation of PKC is also supported by the observation that CaMKII regulates PLA_2 activity [45]. PLA_2 is known to act upstream of PKC in several signaling pathways, including $1\alpha,25(\text{OH})_2\text{D}_3$ and dihydrotestosterone [14,51], hence CaMKII influences PKC activity in a PLA_2 -dependent mechanism. Additionally, in agreement with previous findings [14,51], we found that PLA_2 inhibition abolished Wnt5a induced activation of PKC in a dose-dependent manner.

MC3T3-E1 cells respond to 125 ng/ml Wnt5a with a rapid increase in PKC activity. Here we report that co-treatment with 10^{-10} – 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ abrogates the increase in PKC activation seen with exogenous 125 ng/ml Wnt5a treatment alone, in a dose dependent manner. Co-treatment of osteoblasts with 125 ng/ml Wnt5a and 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$, returned PKC activity to the control level. Previously, we reported that MC3T3-E1 osteoblast-like cells respond to 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ with a rapid increase in PKC activity [9]. However, co-treatment with 50–125 ng/ml Wnt5a leads to a strong increase in PKC activity. Surprisingly, we observed that co-treatment with Wnt5a induces enhanced effects on $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated PKC activation at low dose of Wnt5a and repressive effects at high dose of Wnt5a. These results may suggest that Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ pathways compete at their receptor complex or downstream pathway mediator levels.

Previously, we reported that $1\alpha,25(\text{OH})_2\text{D}_3$ membrane associated receptor, Pdia3, is present in plasma membrane compartments called caveolae [52]. We also showed that PLAA and CaM are present in caveolae, where their interaction with Pdia3 receptor complex is critical for transducing the $1\alpha,25(\text{OH})_2\text{D}_3$ signal [12,13]. In the present study, our plasma membrane fractionation experiment indicates that ROR2, FZD2, and FZD5 are localized in fraction 3. Furthermore, our receptor antibody blocking experiments shows that blocking ROR2 abolishes $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated PKC activation while blocking FZD2 and

FZD5 receptors has no effect on activation of PKC in response to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. These results indicate that Wnt5a co-receptor, ROR2, is part of $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-associate receptor complex in caveolae. In agreement with our findings, another group has detected ROR2 in Cav-1- α positive cholesterol-rich, detergent-resistant microdomains (DRMs) of the plasma membrane in [53]. Furthermore, they showed that ROR2 forms a complex with bone morphogenetic protein receptor type 1B (BMPRI1B) in a ligand-independent manner and it inhibited the growth and differentiation factor 5 (GDF5)/BMPRI1B induced Smad 1/5 signaling pathway in ATDC5 cells [54]. Collectively, these findings suggest that ROR2 participates in multiple signaling pathways including Wnt5a, BMP and $1\alpha,25(\text{OH})_2\text{D}_3$.

Wnt5a induces its non-canonical signaling via several receptors. ROR2 is a transmembrane receptor, previously identified to mediate Wnt5a actions [55]. Wnt5a increases ROR2 expression, and knockdown of WNT5A dramatically decreases expression of ROR2 [30]. ROR2 has regions of cysteine-rich domain that serve as its Wnt binding domain [56, 57]. Several conditions, including brachydactyly type B and autosomal recessive Robinow syndrome, which display severe skeletal dysplasia, are due to mutations in the ROR2 gene [58,59]. Additionally, ROR2 is critically required for Wnt5a-induced migration of osteoblasts [60]. Wnt5a can also act via the Frizzled family of receptors. In human cells, FZD2 and FZD5 are known to act as Wnt5a receptors and activate its non-canonical signaling cascades [61,62].

Our CaM immunoprecipitation studies suggest that CaM forms a complex with ROR2, FZD2, and FZD5. Wnt5a treatment does not alter the interactions between CaM–FZD2 and CaM–FZD5, while it reduces the interaction between CaM and ROR2. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment decreases the interactions between CaM–FZD2 and CaM–ROR2; however, it does not alter the interactions between CaM and FZD5. These results indicate that CaM serves as a mediator in both $1\alpha,25(\text{OH})_2\text{D}_3$ and Wnt5a pathways; suggesting its potential role to mediate the cross-talk between these two pathways.

The results of this study suggest that Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ mediate their effects via a network of interacting mediators rather than through a secluded linear pathway. In co-treatment studies, we speculate that $1\alpha,25(\text{OH})_2\text{D}_3$ receptor competes with Wnt5a receptors for binding of PLAA, CaM and Cav-1, thus inhibiting downstream PKC signaling. Previously, similar $1\alpha,25(\text{OH})_2\text{D}_3$ -induced mechanisms in which one hormone can modulate the activity of a second, by competing for a shared mediator site, have been identified. Work from the Ross lab has shown that retinoid X receptor (RXR)–vitamin D receptor (VDR) and retinoid acid receptor (RAR)–RXR heterodimers compete for a novel steroid hormone response element containing elements responding to retinoid acid and $1\alpha,25(\text{OH})_2\text{D}_3$ in the promoter region of the avian $\beta 3$ integrin gene [63]. Co-treatment with retinoid acid and $1\alpha,25(\text{OH})_2\text{D}_3$ resulted in a response equal to that of retinoid acid alone [63]. Furthermore, their results indicated that RAR–RXR had a

greater affinity for the shared promoter region than RXR–VDR, hence co-addition of retinoid acid and $1\alpha,25(\text{OH})_2\text{D}_3$ resulted in preferred binding of RAR–RXR to the promoter and inhibition of $1\alpha,25(\text{OH})_2\text{D}_3$ induced transcription [63]. Similar to this finding, our results also indicated that Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ compete for similar signaling mediators and co-treatment antagonizes the immunological effects of $1\alpha,25(\text{OH})_2\text{D}_3$. $1\alpha,25(\text{OH})_2\text{D}_3$ is known to stimulate alkaline phosphatase activity [16] and osteopontin production [9], markers of osteoblast maturation, via its membrane-mediated events. Wnt5a has also been shown to promote osteoblast differentiation and maturation. Wnt5a treatment increases osteocalcin and osteoprotegerin levels and alkaline phosphatase specific activity [26], while its knockout down regulates osteoblastic differentiation markers including runt related transcription factor 2, osterix and alkaline phosphatase [27]. Future studies should focus on the in vivo consequences of the regulation of growth plate chondrocytes growth and differentiation by Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$.

In conclusion, this study investigated the requirement for components of $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-associated receptor complex in Wnt5a calcium-dependent signaling. We found that Wnt5a stimulates its calcium-dependent actions via Pdia3 receptor complex (Fig. 8). The effects of Wnt5a are mediated through Pdia3, PLAA, and VDR. Inhibition of CaM, CaMKII, and PLA_2 suppressed the activation of PKC in response to Wnt5a treatment. Silencing Cav-1 had no effect on Wnt5a-stimulated PKC activation, which reveals one of the differences between the mediators of $1\alpha,25(\text{OH})_2\text{D}_3$ and Wnt5a calcium-dependent pathways. Our results also showed that ROR2, one of the receptors of Wnt5a, plays an important role in $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-mediated signaling. Blocking ROR2 abolished $1\alpha,25(\text{OH})_2\text{D}_3$ induced PKC and CaMKII activations. Moreover, immunoprecipitation studies showed that $1\alpha,25(\text{OH})_2\text{D}_3$ membrane receptor complex (Pdia3, PLAA, Cav-1 and CaM) interacts with Wnt5a receptors (ROR2, FZD2 and FZD5). While most of their protein–protein interactions were independent of either $1\alpha,25(\text{OH})_2\text{D}_3$ or Wnt5a treatment, a few of the interactions changed with ligands treatments. In the co-treatment study, addition of $1\alpha,25(\text{OH})_2\text{D}_3$ repressed Wnt5a stimulated PKC activation in a dose-dependent manner. We found that co-treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ repressed Wnt5a induced PKC activation in a dose-dependent manner, and was most inhibited at 10^{-8} M. Furthermore, co-treatment with 50 ng/ml Wnt5a caused a 2-fold increase in $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated PKC activity compared to cultures treated with only $1\alpha,25(\text{OH})_2\text{D}_3$. However, as the concentration of Wnt5a increased, it had repressive effects on $1\alpha,25(\text{OH})_2\text{D}_3$ -induced PKC activation. The results of this study suggest that signaling components of Pdia3 receptor complex are required for mediating the calcium-dependent actions of Wnt5a and $1\alpha,25(\text{OH})_2\text{D}_3$ may modulate the response of Wnt5a by competing for similar signaling mediators.

Acknowledgments

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References

- [1] D.D. Howell DS, Biology, chemistry and biochemistry of the mammalian growth plate, in: F.L. Coe, F. M. (Eds.), Disorders of Bone and Mineral Metabolism, Raven, New York, 1992, pp. 313–353.
- [2] A. Gurlek, R. Kumar, Regulation of osteoblast growth by interactions between transforming growth factor-beta and 1alpha,25-dihydroxyvitamin D3, Crit. Rev. Eukaryot. Gene Expr. 11 (2001) 299–317.
- [3] H.A. Pedrozo, Z. Schwartz, T. Mokeyev, A. Ornoy, W. Xin-Sheng, L.F. Bonewald, D.D. Dean, B.D. Boyan, Vitamin D3 metabolites regulate LTBP1 and latent TGF-beta1 expression and latent TGF-beta1 incorporation in the extracellular matrix of chondrocytes, J. Cell. Biochem. 72 (1999) 151–165.
- [4] J. Alvarez, P. Sohn, X. Zeng, T. Doetschman, D.J. Robbins, R. Serra, TGFbeta2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression, Development 129 (2002) 1913–1924.
- [5] E. Nemoto, Y. Ebe, S. Kanaya, M. Tsuchiya, T. Nakamura, M. Tamura, H. Shimauchi, Wnt5a signaling is a substantial constituent in bone morphogenetic protein-2-mediated osteoblastogenesis, Biochem. Biophys. Res. Commun. 422 (2012) 627–632.
- [6] I. Nemere, A.W. Norman, The rapid, hormonally stimulated transport of calcium (transcaltachia), J. Bone Miner. Res. 2 (1987) 167–169.
- [7] B.D. Boyan, Z. Schwartz, L.D. Swain, L.F. Bonewald, A. Khare, Regulation of matrix vesicle metabolism by vitamin D metabolites, Connect. Tissue Res. 22 (1989) 3–16 (discussion 53–61).
- [8] R.C. Khanal, I. Nemere, The ERp57/Grp58/1,25D3-MARRS receptor: multiple functional roles in diverse cell systems, Curr. Med. Chem. 14 (2007) 1087–1093.
- [9] J. Chen, R. Olivares-Navarrete, Y. Wang, T.R. Herman, B.D. Boyan, Z. Schwartz, Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1,25-dihydroxyvitamin D3 in osteoblasts, J. Biol. Chem. 285 (2010) 37041–37050.
- [10] I. Nemere, M.C. Farach-Carson, B. Rohe, T.M. Sterling, A.W. Norman, B.D. Boyan, S.E. Safford, Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7392–7397.
- [11] C.M. Thomas, E.J. Smart, Caveolae structure and function, J. Cell. Mol. Med. 12 (2008) 796–809.
- [12] M. Doroudi, Z. Schwartz, B.D. Boyan, Phospholipase A(2) activating protein is required for 1alpha,25-dihydroxyvitamin D(3) dependent rapid activation of protein kinase C via Pdia3, J. Steroid Biochem. Mol. Biol. 132 (2012) 48–56.
- [13] M. Doroudi, M.C. Plaisance, B.D. Boyan, Z. Schwartz, Membrane actions of $1\alpha,25(\text{OH})_2\text{D}_3$ is mediated by Ca^{2+} /calmodulin-dependent protein kinase II in bone and cartilage cells, Under Review, 2014.
- [14] V.L. Sylvia, Z. Schwartz, D.B. Curry, Z. Chang, D.D. Dean, B.D. Boyan, $1,25(\text{OH})_2\text{D}_3$ regulates protein kinase C activity through two phospholipid-dependent pathways involving phospholipase A2 and phospholipase C in growth zone chondrocytes, J. Bone Miner. Res. 13 (1998) 559–569.
- [15] Z. Schwartz, D. Shaked, R.R. Hardin, S. Gruwell, D.D. Dean, V.L. Sylvia, B.D. Boyan, $1\alpha,25(\text{OH})_2\text{D}_3$ causes a rapid increase in phosphatidylinositol-specific PLC-beta activity via phospholipase A2-dependent production of lysophospholipid, Steroids 68 (2003) 423–437.
- [16] V.L. Sylvia, F. Del Toro, D.D. Dean, R.R. Hardin, Z. Schwartz, B.D. Boyan, Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on rat growth zone chondrocytes are mediated via cyclooxygenase-1 and phospholipase A(2), J. Cell. Biochem. Suppl. (Suppl. 36) (2001) 32–45.

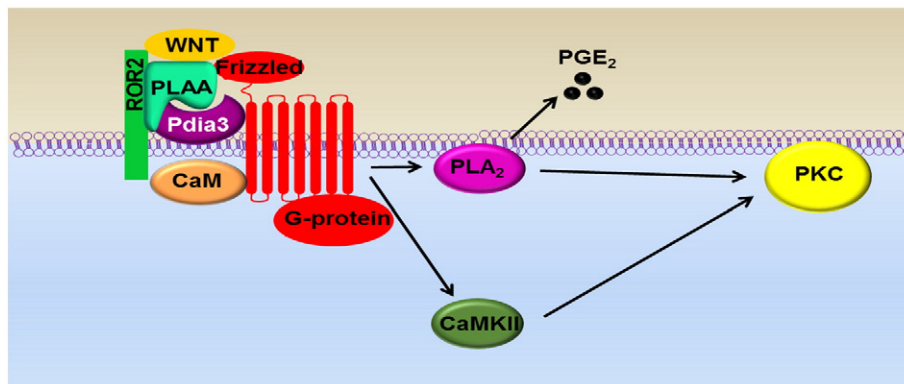


Fig. 8. Proposed role of components of $1\alpha,25(\text{OH})_2\text{D}_3$ membrane-associated receptor complex in Wnt5a calcium-dependent signaling. The effects of Wnt5a are mediated through Pdia3, PLAA and VDR (not shown). Inhibition of CaM, CaMKII, and PLA_2 suppressed the activation of PKC in response to Wnt5a treatment.

- [17] B.D. Boyan, V.L. Sylvia, D.D. Dean, Z. Schwartz, Membrane mediated signaling mechanisms are used differentially by metabolites of vitamin D(3) in musculoskeletal cells, *Steroids* 67 (2002) 421–427.
- [18] A. Kishimoto, Y. Takai, T. Mori, U. Kikkawa, Y. Nishizuka, Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover, *J. Biol. Chem.* 255 (1980) 2273–2276.
- [19] Y. Wang, J. Chen, C.S. Lee, A. Nizkorodov, K. Riemenschneider, D. Martin, S. Hyzy, Z. Schwartz, B.D. Boyan, Disruption of Pdia3 gene results in bone abnormality and affects 1 α ,25-dihydroxy-vitamin D₃-induced rapid activation of PKC, *J. Steroid Biochem. Mol. Biol.* 121 (2010) 257–260.
- [20] H. Coe, J. Jung, J. Groenendyk, D. Prins, M. Michalak, ERp57 modulates STAT3 signaling from the lumen of the endoplasmic reticulum, *J. Biol. Chem.* 285 (2010) 6725–6738.
- [21] I. Nemere, N. Garbi, G.J. Hammerling, R.C. Khanal, Intestinal cell calcium uptake and the targeted knockout of the 1,25D₃-MARRS (membrane-associated, rapid response steroid-binding) receptor/PDIA3/Erp57, *J. Biol. Chem.* 285 (2010) 31859–31866.
- [22] K.E. Yates, S. Shortkroff, R.G. Reish, Wnt influence on chondrocyte differentiation and cartilage function, *DNA Cell Biol.* 24 (2005) 446–457.
- [23] Y. Wang, Y.P. Li, C. Paulson, J.Z. Shao, X. Zhang, M. Wu, W. Chen, Wnt and the Wnt signaling pathway in bone development and disease, *Front. Biosci.* 19 (2014) 379–407.
- [24] A.C. Andrade, O. Nilsson, K.M. Barnes, J. Baron, Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation, *Bone* 40 (2007) 1361–1369.
- [25] Y. Yang, L. Topol, H. Lee, J. Wu, Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation, *Development* 130 (2003) 1003–1015.
- [26] R. Olivares-Navarrete, S.L. Hyzy, D.L. Hutton, G.R. Dunn, C. Appert, B.D. Boyan, Z. Schwartz, Role of non-canonical Wnt signaling in osteoblast maturation on microstructured titanium surfaces, *Acta Biomater.* 7 (2011) 2740–2750.
- [27] J. Guo, J. Jin, L.F. Cooper, Dissection of sets of genes that control the character of wnt5a-deficient mouse calvarial cells, *Bone* 43 (2008) 961–971.
- [28] X. He, J.P. Saint-Jeannet, Y. Wang, J. Nathans, I. Dawid, H. Varmus, A member of the Frizzled protein family mediating axis induction by Wnt-5A, *Science* 275 (1997) 1652–1654.
- [29] A.V. Bazhin, V. Tambor, B. Dikov, P.P. Philippov, D. Schadendorf, S.B. Eichmuller, cGMP-phosphodiesterase 6, transducin and Wnt5a/Frizzled-2-signaling control cGMP and Ca(2+) homeostasis in melanoma cells, *Cell. Mol. Life Sci.* 67 (2010) 817–828.
- [30] M.P. O'Connell, J.L. Fiori, M. Xu, A.D. Carter, B.P. Frank, T.C. Camilli, A.D. French, S.K. Dissanayake, F.E. Indig, M. Bernier, D.D. Taub, S.M. Hewitt, A.T. Weeraratna, The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma, *Oncogene* 29 (2010) 34–44.
- [31] A. Kikuchi, H. Yamamoto, A. Sato, S. Matsumoto, Wnt5a: its signalling, functions and implication in diseases, *Acta Physiol.* 204 (2012) 17–33.
- [32] M. Kuhl, L.C. Sheldahl, C.C. Malbon, R.T. Moon, Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in *Xenopus*, *J. Biol. Chem.* 275 (2000) 12701–12711.
- [33] H.A. Pedrozo, Z. Schwartz, S. Rimes, V.L. Sylvia, I. Nemere, G.H. Posner, D.D. Dean, B.D. Boyan, Physiological importance of the 1,25(OH)₂D₃ membrane receptor and evidence for a membrane receptor specific for 24,25(OH)₂D₃, *J. Bone Miner. Res.* 14 (1999) 856–867.
- [34] J. Chen, M. Doroudi, J. Cheung, A.L. Grozier, Z. Schwartz, B.D. Boyan, Plasma membrane Pdia3 and VDR interact to elicit rapid responses to 1 α ,25(OH)₂D₃, *Cell. Signal.* 25 (2013) 2362–2373.
- [35] D. Wang, K. Christensen, K. Chawla, G. Xiao, P.H. Krebsbach, R.T. Franceschi, Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential, *J. Bone Miner. Res.* 14 (1999) 893–903.
- [36] B.D. Boyan, Z. Schwartz, L.D. Swain, D.L. Carnes Jr., T. Zisli, Differential expression of phenotype by resting zone and growth region costochondral chondrocytes in vitro, *Bone* 9 (1988) 185–194.
- [37] Z. Schwartz, E.J. Graham, L. Wang, S. Lossdorfer, I. Gay, T.L. Johnson-Pais, D.L. Carnes, V.L. Sylvia, B.D. Boyan, Phospholipase A2 activating protein (PLAA) is required for 1 α ,25(OH)₂D₃ signaling in growth plate chondrocytes, *J. Cell. Physiol.* 203 (2005) 54–70.
- [38] Y. Liu, B. Rubin, P.V. Bodine, J. Billiard, Wnt5a induces homodimerization and activation of Ror2 receptor tyrosine kinase, *J. Cell. Biochem.* 105 (2008) 497–502.
- [39] Z. Schwartz, H. Ehland, V.L. Sylvia, D. Larsson, R.R. Hardin, V. Bingham, D. Lopez, D.D. Dean, B.D. Boyan, 1 α ,25-dihydroxyvitamin D(3) and 24R,25-dihydroxyvitamin D(3) modulate growth plate chondrocyte physiology via protein kinase C-dependent phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase, *Endocrinology* 143 (2002) 2775–2786.
- [40] B.D. Boyan, K.L. Wong, L. Wang, H. Yao, R.E. Guldberg, M. Drab, H. Jo, Z. Schwartz, Regulation of growth plate chondrocytes by 1,25-dihydroxyvitamin D₃ requires caveolae and caveolin-1, *J. Bone Miner. Res.* 21 (2006) 1637–1647.
- [41] C. Nebigil, K.U. Malik, Alpha adrenergic receptor subtypes involved in prostaglandin synthesis are coupled to Ca⁺⁺ channels through a pertussis toxin-sensitive guanine nucleotide-binding protein, *J. Pharmacol. Exp. Ther.* 266 (1993) 1113–1124.
- [42] B.H. Chang, S. Mukherji, T.R. Soderling, Characterization of a calmodulin kinase II inhibitor protein in brain, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 10890–10895.
- [43] M. Murakami, H. Kuwata, Y. Amakasu, S. Shimbara, Y. Nakatani, G. Atsumi, I. Kudo, Prostaglandin E2 amplifies cytosolic phospholipase A2- and cyclooxygenase-2-dependent delayed prostaglandin E2 generation in mouse osteoblastic cells. Enhancement by secretory phospholipase A2, *J. Biol. Chem.* 272 (1997) 19891–19897.
- [44] J.A. Huhtakangas, C.J. Olivera, J.E. Bishop, L.P. Zanello, A.W. Norman, The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 α ,25(OH)₂-vitamin D₃ in vivo and in vitro, *Mol. Endocrinol.* 18 (2004) 2660–2671.
- [45] M.M. Muthalif, I.F. Benter, M.R. Uddin, K.U. Malik, Calcium/calmodulin-dependent protein kinase II α mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells, *J. Biol. Chem.* 271 (1996) 30149–30157.
- [46] L. Li, B.I. Hutchins, K. Kalil, Wnt5a induces simultaneous cortical axon outgrowth and repulsive axon guidance through distinct signaling mechanisms, *J. Neurosci.* 29 (2009) 5873–5883.
- [47] J. Chen, M. Doroudi, J. Cheung, A.L. Grozier, Z. Schwartz, B.D. Boyan, Plasma membrane Pdia3 and VDR interact to elicit rapid responses to 1 α ,25(OH)₂D(3), *Cell Signal.* 25 (12) (2013) 2362–2373.
- [48] A.W. Norman, Minireview: vitamin D receptor: new assignments for an already busy receptor, *Endocrinology* 147 (2006) 5542–5548.
- [49] H. Yamamoto, H. Komekado, A. Kikuchi, Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin, *Dev. Cell* 11 (2006) 213–223.
- [50] J.K. Chuprun, E. Bazan, K.C. Chang, A.K. Campbell, R.M. Rapoport, Inhibition of phorbol ester-induced contraction by calmodulin antagonists in rat aorta, *Am. J. Physiol.* 261 (1991) C675–C684.
- [51] K. ElBaradie, Y. Wang, B.D. Boyan, Z. Schwartz, Rapid membrane responses to dihydrotestosterone are sex dependent in growth plate chondrocytes, *J. Steroid Biochem. Mol. Biol.* 132 (2012) 15–23.
- [52] J. Chen, K.S. Lobachev, B.J. Grindel, M.C. Farach-Carson, S.L. Hyzy, K.B. El-Baradie, R. Olivares-Navarrete, M. Doroudi, B.D. Boyan, Z. Schwartz, Chaperone properties of pdia3 participate in rapid membrane actions of 1 α ,25-dihydroxyvitamin D₃, *Mol. Endocrinol.* 27 (2013) 1065–1077.
- [53] M. Sammar, C. Sieber, P. Knaus, Biochemical and functional characterization of the Ror2/BRIb receptor complex, *Biochem. Biophys. Res. Commun.* 381 (2009) 1–6.
- [54] M. Sammar, S. Stricker, G.C. Schwabe, C. Sieber, A. Hartung, M. Hanke, I. Oishi, J. Pohl, Y. Minami, W. Seibald, S. Mundlos, P. Knaus, Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2, *Genes Cells* 9 (2004) 1227–1238.
- [55] I. Oishi, H. Suzuki, N. Onishi, R. Takada, S. Kani, B. Ohkawara, I. Koshida, K. Suzuki, G. Yamada, G.C. Schwabe, S. Mundlos, H. Shibuya, S. Takada, Y. Minami, The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway, *Genes Cells* 8 (2003) 645–654.
- [56] R. Cheung, J. Kelly, R.J. Macleod, Regulation of villin by wnt5a/ror2 signaling in human intestinal cells, *Front. Physiol.* 2 (2011) 58.
- [57] J. Saldanha, J. Singh, D. Mahadevan, Identification of a Frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases, *Protein Sci.* 7 (1998) 1632–1635.
- [58] A.R. Afzal, A. Rajab, C.D. Fenske, M. Oldridge, N. Elanko, E. Ternes-Pereira, B. Tuysuz, V.A. Murday, M.A. Patton, A.O. Wilkie, S. Jeffery, Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2, *Nat. Genet.* 25 (2000) 419–422.
- [59] H. van Bokhoven, J. Celli, H. Kayserili, E. van Beusekom, S. Balci, W. Brussel, F. Skovby, B. Kerr, E.F. Percin, N. Akarsu, H.G. Brunner, Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome, *Nat. Genet.* 25 (2000) 423–426.
- [60] M. Nishita, S.K. Yoo, A. Nomachi, S. Kani, N. Sougawa, Y. Ohta, S. Takada, A. Kikuchi, Y. Minami, Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration, *J. Cell Biol.* 175 (2006) 555–562.
- [61] H. Wang, Y. Lee, C.C. Malbon, PDE6 is an effector for the Wnt/Ca²⁺/cGMP-signalling pathway in development, *Biochem. Soc. Trans.* 32 (2004) 792–796.
- [62] A. Blumenthal, S. Ehlers, J. Lauber, J. Buer, C. Lange, T. Goldmann, H. Heine, E. Brandt, N. Reiling, The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation, *Blood* 108 (2006) 965–973.
- [63] X. Cao, S.L. Teitelbaum, H.J. Zhu, L. Zhang, X. Feng, F.P. Ross, Competition for a unique response element mediates retinoic acid inhibition of vitamin D₃-stimulated transcription, *J. Biol. Chem.* 271 (1996) 20650–20654.