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Vitamin D Receptor is not Required for the Rapid Actions of 1,25-Dihydroxyvitamin D_3 to Increase Intracellular Calcium and Activate Protein Kinase C in Mouse Osteoblasts

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Abstract The rapid, non-genomic actions of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] have been well described, however, the role of the nuclear vitamin D receptor (VDR) in this pathway remains unclear. To address this question, we used VDR(+/+) and VDR(−/−) osteoblasts isolated from wild-type and VDR null mice to study the increase in intracellular calcium ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC) induced by 1,25$(OH)_2D_3$. Within 1 min of 1,25$(OH)_2D_3$ (100 nM) treatment, an increase of 58 and 53 nM in $[Ca^{2+}]_i$ (n = 3) was detected in VDR(+/+) and VDR(−/−) cells, respectively. By 5 min, 1,25$(OH)_2D_3$ caused a 2.1- and 1.9-fold increase (n = 6) in the phosphorylation of PKC substrate peptide acetylated-MBP_{4-14} in VDR(+/+) and VDR(−/−) osteoblasts. The 1,25$(OH)_2D_3$-induced phosphorylation was abolished by GF109203X, a general PKC inhibitor, in both cell types, confirming that the secosteroid induced PKC activity. Moreover, 1,25$(OH)_2D_3$ treatment resulted in the same degree of translocation of PKC-α and PKC-δ, but not of PKC-ζ, from cytosol to plasma membrane in both VDR(+/+) and VDR(−/−) cells. These experiments demonstrate that the 1,25$(OH)_2D_3$-induced rapid increases in $[Ca^{2+}]_i$ and PKC activity are neither mediated by, nor dependent upon, a functional nuclear VDR in mouse osteoblasts. Thus, VDR is not essential for these rapid actions of 1,25$(OH)_2D_3$ in osteoblasts. J. Cell. Biochem. 88: 794–801, 2003.

Key words: vitamin D; VDR; non-genomic actions; protein kinase C; calcium

The biological effects of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] are characterized by nuclear transcriptional regulation of gene expression as well as by rapid, non-genomic effects. The classical transcriptional activity of 1,25$(OH)_2D_3$ is believed to be initiated by its interaction with the vitamin D receptor (VDR) in the cytosol, followed by nuclear translocation of the liganded receptor, which then interacts with vitamin D response elements in the promoter region of target genes, ultimately leading to changes in gene expression in a time frame of hours [Haussler et al., 1998]. The rapid, non-genomic actions of 1,25$(OH)_2D_3$, on the other hand, occur rapidly in seconds to minutes and thus are incompatible with mechanisms involving alterations in gene transcription and protein synthesis. The non-genomic effects, which have been reported in many cell types, include rapid changes in intracellular calcium concentrations ($[Ca^{2+}]_i$), alterations in membrane phospholipid metabolism, and activation of protein kinase C (PKC) [Nemere and Norman, 1988; Caffrey and Farach-Carson, 1989; Baum et al., 1990; Wali et al., 1990; Simboli-Campbell et al., 1994; Slater et al., 1995; Berry et al., 1996; Zanello and Norman, 1996, 1997; Norman, 1997, 1998]. However, the mechanism that mediates the non-genomic actions of 1,25$(OH)_2D_3$ remains unclear. In particular, the involvement of the VDR in this pathway remains highly controversial. On the one hand, there is evidence that supports the existence of membrane receptor(s) other than VDR for the rapid response. For instance, the rapid effect of

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$1,25(OH)_2D_3$ on PKC activity was seen in cells treated with vitamin D analogs with low binding affinity to VDR [Nemere and Norman, 1988; Boyan et al., 1997], and a rapid increase in $[Ca^{2+}]_i$ was seen in $1,25(OH)_2D_3$-treated cells in which VDR expression was undetectable [Baran et al., 1991]. Furthermore, some membrane-bound proteins have been identified as putative receptors to mediate the rapid responses of $1,25(OH)_2D_3$ [Nemere et al., 1994, 1998; Baran et al., 2000], and PKC itself has been suggested to be a membrane bound receptor for $1,25(OH)_2D_3$ [Slater et al., 1995]. On the other hand, VDR may also act as a cytosolic receptor to mediate the non-genomic effects. Early data showed that transient exposure to $1,25(OH)_2D_3$ stimulated the accumulation of cGMP around VDR [Barsony and Marx, 1991]. More recent studies demonstrated that the nuclear VDR was translocated to the microsomal membrane fraction as a result of rapid $1,25(OH)_2D_3$ stimulation in chick muscle cells [Boland et al., 2002; Capiati et al., 2002], and that the $1,25(OH)_2D_3$-induced rapid increase in intracellular calcium was abrogated in cells lacking VDR [Erben et al., 2002], suggesting that VDR may mediate the non-genomic effects.

To address whether VDR is involved in the non-genomic effects of $1,25(OH)_2D_3$, in this report we studied the rapid effects of this secosteroid in VDR(−/−) osteoblasts isolated from VDR knockout mice that lack a functional VDR [Li et al., 1997]. We chose to study cultured calvaria osteoblasts because this is a well-established model system, and specific membrane binding activity of $1,25(OH)_2D_3$ has been reported in osteoblast-like cells [Baran et al., 1994]. We used the increase in $[Ca^{2+}]_i$ and PKC activity and translocation as the relevant parameters to assess the rapid effects of $1,25(OH)_2D_3$. Our data demonstrate that VDR is not required for these rapid effects of $1,25(OH)_2D_3$ in mouse osteoblasts.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ was kindly provided by Dr. M.R. Uskokovic (Hoffman LaRoche, Inc., Nutley, NJ), and stock solutions were prepared in ethanol and maintained in the dark under N_2 at 4°C. Protease inhibitor cocktail tablets (complete) were purchased from Roche Diagnostics Corp. (Indianapolis, IN). PKC inhibitor GF109203X was obtained from LC Laboratories (Woburn, MA). PKC substrate peptide derived from myelin basic protein (acetylated-MBP_{4-14}), phosphocellulose disks, TRIzol reagent, and cell culture media were obtained from Invitrogen-Life Technologies (Grand Island, NY). Monoclonal anti-PKC-α antibody was obtained from Upstate Biotechnology (Lake Placid, NY), and polyclonal anti-PKC-δ and -ζ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoretic grade acrylamide, bis-acrylamide, Tris, SDS, and prestained molecular weight markers were obtained from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes (Immobilon-P) were purchased from Millipore (Bradford, MA). The enhanced chemiluminescent substrate kit, the peroxidase-coupled sheep anti-mouse, and donkey anti-rabbit antibodies and [γ^{32}-P]ATP were supplied by Amersham (Arlington Heights, IL). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). EDTA, EGTA, ATP, ammonium persulfate, TEMED, and 2-mercaptoethanol were purchased from Sigma Chemical (St. Louis, MO). X-OMAT AR films were from Kodak (Rochester, NY). Recombinant TGF-β1 was obtained from R&D System (Minneapolis, MN).

Isolation and Culture of Osteoblasts

Osteoblasts were isolated from calvaria of 3-day-old wild-type (+/+) and VDR null (−/−) mice by sequential collagenase digestion as described previously [Wong and Cohn, 1974]. VDR(+/+) and VDR(−/−) pups were identified by PCR using the tail genomic DNA as the template as described previously [Li et al., 1997]. The cells were then cultured in alpha-MEM Eagle supplemented with 10% FBS and split once at confluence. Osteoblasts grown from the first passage were used in all experiments.

RNA Isolation and Northern Blot

Osteoblasts isolated from wild-type and VDR(−/−) mice were grown in 10-cm dishes to 80% confluency. To examine osteopontin (OP) expression, the cells were treated with vehicle or 10 nM of $1,25(OH)_2D_3$ for 24 h in serum-free medium. To examine interleukin-1α (IL-1α) expression, the cells were treated with 2 ng/ml of TGF-β1 for 4 and 6 h in serum-free medium. Total cellular RNA was then extracted by using TRIzol Reagent (Invitrogen-Life Technologies) according to the manufacturer's instructions.

For Northern blot analyses, total RNAs (20 μg/lane) were separated on a 1.2% agarose gel and transferred onto a nylon membrane. The membranes were then hybridized with ^{32}P-labeled OP, IL-1α, or actin cDNA probe as described previously [Li et al., 2001]. The cDNA probes were labeled with the Prime-a-Gene Labeling Kit from Promega (Madison, WI).

Measurement of Intracellular Calcium

For $[Ca^{2+}]_i$ measurements, VDR(+/+) and (−/−) osteoblasts grown on coverslips were loaded with 5 μM Fura-2/AM for 30 min at room temperature. The cells were rinsed in PBS and incubated in the buffer containing 135 mM NaCl, 6 mM KCl, 20 mM HEPES (pH 7.2), 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% BSA, and 2.8 mM D-glucose. The change of fluorescence intensity at 340 and 380 nm in the Fura-2-loaded osteoblasts before and after $1,25(OH)_2D_3$ (100 nM) treatment was recorded and analyzed using a DELTSCAN (Photon Technology, Jersey City, NJ). The ratios of fluorescence intensity at 340 and 380 nM were determined, and the values of $[Ca^{2+}]_i$ were calculated based on the formula of Grynkiewicz et al. [1985] as previously described [Bissonnette et al., 1994].

In Situ Assay of PKC Activity

The activity of PKC was determined by measuring the phosphorylation of a PKC peptide substrate. Briefly, VDR(+/+) and (−/−) osteoblasts were grown to 80–85% confluency in 24-well plates. The cells were rinsed twice and treated in serum-free alpha-MEM Eagle with 10 nM of $1,25(OH)_2D_3$ for indicated times, or with the indicated concentrations of $1,25(OH)_2D_3$ for 5 min. In other experiments, the cells were pretreated with a PKC inhibitor or vehicle for 3 h before being stimulated with $1,25(OH)_2D_3$. After aspiration of the medium, PKC activity was assayed by incubating the cells for 10 min in 100 μl of permeabilization-kinase assay buffer per well as previously described [Khare et al., 1999]. The buffer contained 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 1 mg/ml glucose, 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 2.5 mM $CaCl_2$, 5 mM EGTA, 100 μM [γ^{32}-P]-ATP (500 cpm/pmol), 50 μg/ml digitonin, and 50 μM PKC substrate peptide acetylated-MBP_{4-14}. The reaction was terminated by addition of 10 μl of 25% TCA (wt/vol) and an aliquot was spotted onto a phosphocellulose disk, which was then washed with 1% (vol/vol) phosphoric acid and counted in a scintillation counter.

Translocation of PKC Isoforms

To examine the translocation of PKC isoforms, VDR(+/+) and (−/−) osteoblasts were grown in 100 mm dishes to 80–85% confluency. The cells were washed and preincubated for 30 min at 37°C in 2 ml of buffer containing 10 mM HEPES, pH 7.2, 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM glucose. The cells were then incubated with 10 nM of $1,25(OH)_2D_3$. At the indicated times, the cells were lysed in 0.5 ml of extraction buffer by three cycles of freezing in a dry ice–ethanol bath, followed by thawing in a 37°C water bath. The extraction buffer contained 25 mM Tris (pH 7.5), 5 mM EGTA, 0.7 mM $CaCl_2$, and one tablet of Complete (protease inhibitor cocktail tablet) in 50 ml buffer. The cells were further disrupted by three passages through 18–25-gauge needles and fractionated into soluble and particulate components by centrifugation at 100,000*g* for 30 min at 4°C in a Sorvall-RCM 120 EX ultracentrifuge. Fractions were immediately boiled in Laemmli SDS-stop buffer. Proteins (30 μg/lane) were separated by SDS–PAGE using a 10% resolving polyacrylamide gel [Laemmli, 1970] and electroblotted onto an Immobilon-P membrane. Blots were incubated overnight at 4°C with specific primary antibodies [mouse monoclonal anti-PKC-α antibody (0.1 μg/ml) or polyclonal anti-PKC-δ or -ζ antibody (0.2 μg/ml)], followed by incubation with appropriate peroxidase-coupled secondary antibodies and subsequent detection by enhanced chemiluminescence as described previously [Bissonnette et al., 1994; Li et al., 2001].

RESULTS

Characterization of Osteoblasts Isolated From Wild-Type and VDR Knockout Mice

The expression of VDR in osteoblasts was examined by Western blot analysis with an anti-VDR antibody. As expected, VDR protein was detected in VDR (+/+) osteoblasts, but not in VDR(−/−) osteoblasts (Fig. 1A). The basal mRNA level of OP, a well-known vitamin D target gene, was comparable in VDR(+/+) and VDR(−/−) osteoblasts. When the cells were treated with $1,25(OH)_2D_3$ for 24 h, however, a significant induction of OP expression was seen in VDR(+/+), but not in VDR(−/−) cells

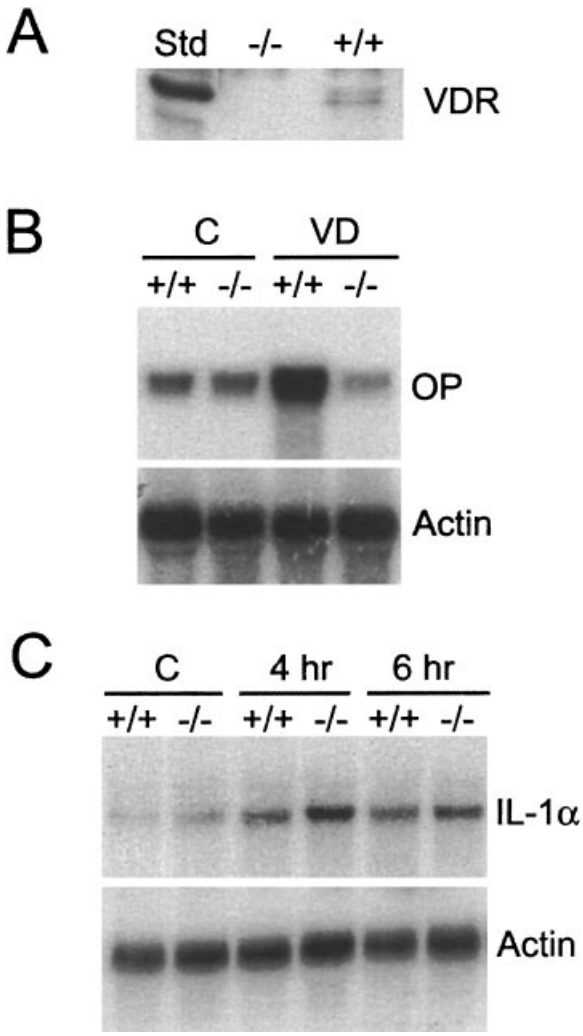

Fig. 1. Characterization of osteoblasts isolated from wild-type (+/+) and VDR(−/−) mice. **A**: Western blot analysis of cell lysates of VDR(+/+) and VDR(−/−) osteoblasts with anti-VDR antibody. Each lane was loaded with 30 μg protein. Std, human VDR standard. Note the VDR protein was detected as a doublet with this antibody. **B**: Northern blot analysis (20 μg total RNA/lane) of osteopontin (OP) mRNA expression in VDR(+/+) and VDR(−/−) osteoblasts treated with 10 nM of $1,25(OH)_2D_3$ for 24 h. C, ethanol-treated control; VD, $1,25(OH)_2D_3$-treated. **C**: Northern blot analysis of IL-1α mRNA expression in VDR(+/+) and VDR(−/−) osteoblasts treated with 2 ng/ml of TGF-β1 for 4 and 6 h. C, untreated control; 4 and 6 h, treated with TGF-β1 for 4 and 6 h. Hybridization with mouse actin cDNA probe serves as an internal loading control.

(Fig. 1B), confirming the lack of VDR-mediated transcription in VDR(−/−) osteoblasts. In contrast, when the cells were treated with TGF-β1, both VDR(+/+) and VDR(−/−) osteoblasts showed a significant induction of IL-1α mRNA expression (Fig. 1C). Thus, despite the lack of VDR, the VDR(−/−) osteoblasts appeared to be functionally normal with respect to other signaling pathways.

Rapid Rise in Intracellular Calcium Concentration in VDR(+/+) and VDR(−/−) Osteoblasts Treated With $1,25(OH)_2D_3$

Fura-2/AM was used to monitor the change of $[Ca^{2+}]_i$ in VDR(+/+) and VDR(−/−) osteoblasts treated with $1,25(OH)_2D_3$. As shown in Figure 2, $1,25(OH)_2D_3$ treatment significantly increased $[Ca^{2+}]_i$ from 61 ± 25 to 119 ± 45 nM in VDR(+/+) cells, and from 49 ± 15 to 102 ± 41 nM in VDR(−/−) cells within 1 min. There was no significant difference in the baseline $[Ca^{2+}]_i$ (61 ± 25 vs. 49 ± 15 nM) and the net increase of $[Ca^{2+}]_i$ (58 vs. 53 nM) between VDR(+/+) and VDR(−/−) cells. Thus, VDR is not required for the $1,25(OH)_2D_3$-induced rapid increase of $[Ca^{2+}]_i$.

$1,25(OH)_2D_3$ Rapidly Induces PKC Activity in Both VDR(+/+) and VDR(−/−) Osteoblasts

To investigate the effect of $1,25(OH)_2D_3$ on PKC activity, osteoblasts were treated with 10 nM of $1,25(OH)_2D_3$ for different times, and the kinase activity was measured by the phosphorylation of a myelin basic protein-derived

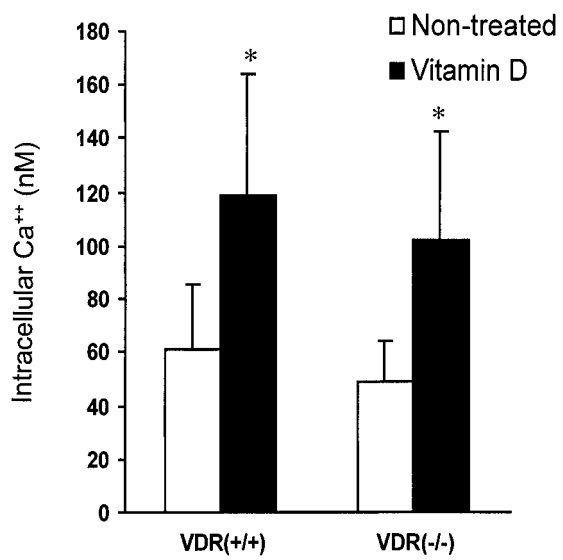

Fig. 2. Effect of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in VDR(+/+) and VDR(−/−) osteoblasts. Primary osteoblasts, loaded with 5 μM of Fura 2-AM, were treated with 100 nM of $1,25(OH)_2D_3$. The fluorescence intensity was measured and recorded with a DELTASCAN for 3 min. The changes in $[Ca^{2+}]_i$ before and after $1,25(OH)_2D_3$ treatment were calculated based on the formula of Grynkiewicz et al. as described in Materials and Methods. Values are mean ± SD. $^*P < 0.05$ vs. non-treated control; n = 3.

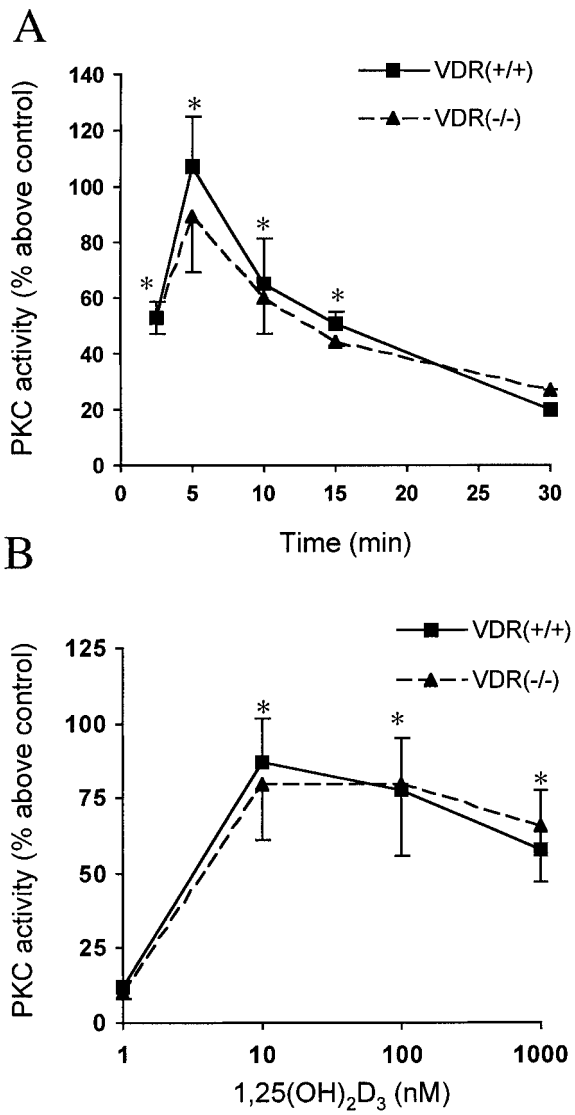

Fig. 3. Effect of $1,25(OH)_2D_3$ on PKC enzyme activity in VDR(+/+) and VDR(−/−) osteoblasts. **A**: Time course effect of $1,25(OH)_2D_3$ on PKC activity. VDR(+/+) and VDR(−/−) osteoblasts were treated with 10 nM of $1,25(OH)_2D_3$ for indicated times and the kinase activity was measured by the phosphorylation of the myelin basic protein-derived peptide (acetylated-MBP_{4-14}) in the presence of [γ^{32}-P]-ATP as described in Materials and Methods. **B**: Effect of different doses of $1,25(OH)_2D_3$ on PKC activity. VDR(+/+) and VDR(−/−) osteoblasts were treated with indicated concentrations of $1,25(OH)_2D_3$ for 5 min and the kinase activity was determined as described in A. Values are mean ± SD. $^*P < 0.05$ vs. untreated control cells; n = 6.

peptide, acetylated-MBP_{4-14}, in the presence of [γ^{32}-P]-ATP. As shown in Figure 3A, the stimulation of acetylated-MBP_{4-14} phosphorylation by $1,25(OH)_2D_3$ was rapid and transient in both VDR(+/+) and VDR(−/−) cells, peaking at 5 min, with a 2.1-fold increase in kinase activity seen in VDR(+/+) osteoblasts, and a 1.9-fold increase in VDR(−/−) cells. Moreover, as shown in Figure 3B, the maximal induction of the kinase activity was observed in VDR(+/+) and VDR(−/−) cells at 10 nM of $1,25(OH)_2D_3$, with no further increase at higher concentrations up to 1,000 nM. To confirm that the increased phosphorylation of acetylated-MBP_{4-14} was mediated by PKC, we examined the ability of GF109203X, a broad spectrum PKC inhibitor [Toullec et al., 1991], to block this phosphorylation induced by $1,25(OH)_2D_3$. As shown in Figure 4, GF109203X significantly inhibited the $1,25(OH)_2D_3$-induced stimulation of kinase activity in both cell types, indicating that $1,25(OH)_2D_3$ activated PKC.

$1,25(OH)_2D_3$ Activates the Same PKC Isoforms in VDR(+/+) and VDR(−/−) Osteoblasts

In many cells, including osteoblasts, PKC activation is associated with translocation of PKC protein from the cytosol to the membrane fraction. We thus investigated whether $1,25(OH)_2D_3$ activated the same specific PKC isoforms in both VDR(+/+) and VDR(−/−) cells by assessing PKC translocation as we previously described [Bissonnette et al., 1994]. To this end, lysates from $1,25(OH)_2D_3$-treated cells were analyzed by quantitative Western blot using antibodies specific for PKC-α, -δ, and -ζ

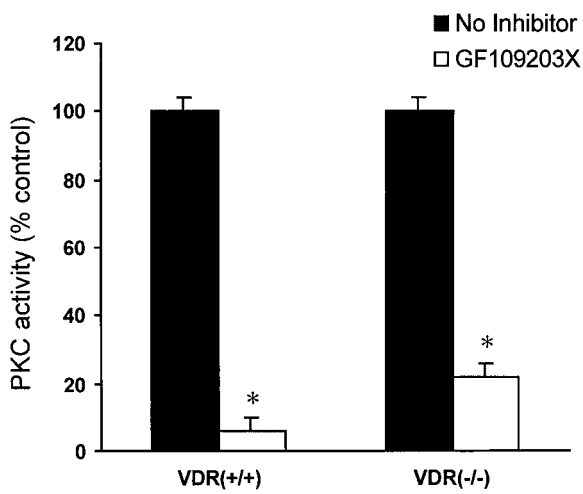

Fig. 4. Effect of a specific PKC inhibitor on $1,25(OH)_2D_3$-induced activation of PKC in VDR(+/+) and VDR(−/−) osteoblasts. Cells were pretreated for 3 h with or without 5 mM of GF109203X, and then stimulated with 10 nM of $1,25(OH)_2D_3$ for 5 min. The kinase activity was measured by the phosphorylation of the myelin basic protein-derived peptide (acetylated-MBP_{4-14}) in the presence of [γ^{32}-P]ATP. Values are mean ± SD. $^*P < 0.05$ vs. cells without the inhibitor treatment; n = 6.

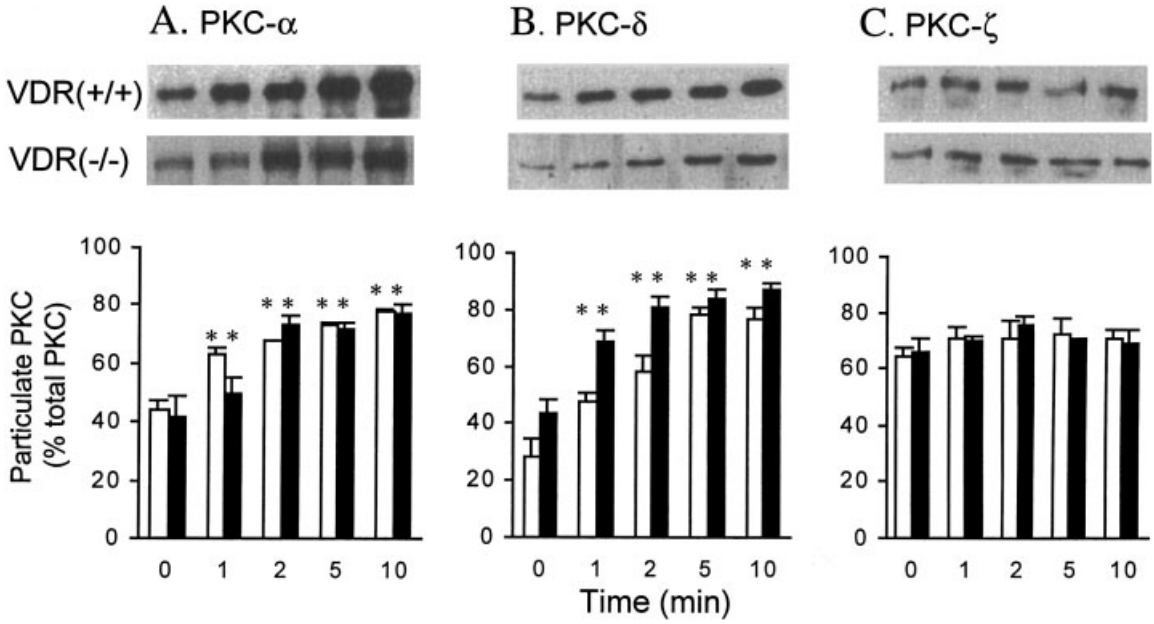

Fig. 5. Differential effects of $1,25(OH)_2D_3$ on the translocation of PKC isoforms in VDR(+/+) and VDR(−/−) osteoblasts. VDR(+/+) and VDR(−/−) cells were treated with 10 nM of $1,25(OH)_2D_3$ for the indicated times, and total cell lysates and particulate fractions were subject to Western blot analyses with PKC isoform-specific antibodies. **A**: Anti-PKC-α antibody. **B**: Anti-PKC-δ antibody. **C**: Anti-PKC-ζ antibody. **Upper panels**: representative Western blots of the indicated PKC isoforms in the particulate fraction. **Lower panels**: quantitation of the Western blots. The relative amount of a PKC isoform in the particulate was presented as a percentage of this isoform in the total cell lysates. Open box, VDR(+/+); filled box, VDR(−/−). Values are mean ± SD. $^*P < 0.05$ vs. untreated cells at 0 min; n = 4.

isoforms. As shown in Figure 5, in both VDR(+/+) and VDR(−/−) osteoblasts, $1,25(OH)_2D_3$ treatment caused the same level of cytosol to membrane translocation for PKC-α and PKC-δ but not for PKC-ζ. These results demonstrate that the rapid increase in PKC activity and subcellular translocation induced by $1,25(OH)_2D_3$ is independent of VDR in osteoblasts.

DISCUSSION

Although the rapid, non-genomic effects of $1,25(OH)_2D_3$ have been documented in a variety of cell types, it is unclear whether the nuclear VDR is directly involved in the process. In part, this reflects the difficulty of distinguishing the effects of the VDR from those of other putative receptors, even in cells with undetectable VDR [Baran et al., 1991]. The generation of transgenic mice, in which the VDR gene has been genetically ablated, has made it possible to study cells that completely lack the nuclear receptor. In the present study, we used osteoblasts derived from VDR knockout mice to address this question. We first confirmed the lack of VDR protein and VDR-mediated transcriptional regulation in these VDR(−/−) cells, which are otherwise normal in other signaling pathways. Then we showed that VDR(−/−) osteoblasts behave exactly like wild-type osteoblasts in response to transient $1,25(OH)_2D_3$ treatment, which includes a rapid increase in intracellular calcium concentration and rapid enzymatic activation and protein translocation of PKC. These results demonstrate convincingly that VDR is not required for the rapid effects of $1,25(OH)_2D_3$ in mouse osteoblasts.

Indeed, previous studies have provided strong evidence for VDR-independence of the non-genomic effects and for the existence of membrane receptor(s) for $1,25(OH)_2D_3$. For instance, $1,25(OH)_2D_3$ rapidly stimulates polyphosphoinositide hydrolysis, raises the $[Ca^{2+}]_i$, and activates PKC in rat colonic epithelial cells [Wali et al., 1990]. $1,25(OH)_2D_3$ primes NB4 cells, an acute promyelocytic leukemia cell line, for monocytic differentiation by a non-genomic pathway that is independent of VDR binding, but dependent on the activation of PKC-α and -δ [Bhatia et al., 1995, 1996]. Rapid effects of $1,25(OH)_2D_3$ were seen in chondrocyte plasma membrane and osteosarcoma cells lacking detectable VDR [Schwartz et al., 1988; Baran

et al., 1991]. Vitamin D analogues with low affinity to VDR were reported to exert rapid effects on membrane phosphoinositide turnover, $[Ca^{2+}]_i$, and PKC [Tien et al., 1993; Greising et al., 1997]. Moreover, a direct association of $1,25(OH)_2D_3$ with plasma membrane binding sites has been described [Baran et al., 1994; Kim et al., 1996], and more recently, putative membrane receptors for $1,25(OH)_2D_3$ have been identified [Nemere et al., 1998; Baran et al., 2000]. Consistent with these studies, our results provide convincing evidence for the hypothesis that the non-genomic effects of $1,25(OH)_2D_3$ are mediated by VDR-independent mechanisms.

Given the complexity of $1,25(OH)_2D_3$ non-genomic effects, however, it should be emphasized that our study only proves that the rapid effect of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ and PKC in mouse osteoblasts is independent of VDR. It does not exclude the possibility that VDR may be directly involved in the non-genomic effects in other cell types, or the possibility of VDR involvement in rapid effects other than changes in $[Ca^{2+}]_i$ and PKC in osteoblasts. In this regard, $1,25(OH)_2D_3$-induced VDR translocation to the microsomal membrane has been reported recently in skeletal muscle cells, suggesting the involvement of VDR in mediating the non-genomic effects of $1,25(OH)_2D_3$ [Capiati et al., 2002]. It should also be pointed out that several lines of VDR knockout mice have been generated, and a recent study reported that the rapid effect of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ was abolished in osteoblasts derived from another line of VDR knockout mice, but the rapid effect on PKC was not reported [Erben et al., 2002]. The reason for this discrepant result from our study is not known.

Data from the present study, and from other groups, suggest that a number of cell types, including osteoblasts, chondrocytes, and intestinal cells, may be subject to genomic and non-genomic vitamin D regulation that involves distinct nuclear and membrane receptors [Norman, 1998]. The ability of $1,25(OH)_2D_3$ to interact with these receptors may reside in the conformational flexibility of the $1,25(OH)_2D_3$ molecule. It has been proposed, for example, that a modified 6-*s-trans* bowl-shape of the molecule binds to the nuclear receptor, while the rapid effects result from an interaction of a planar 6-*s-cis* ligand shape of the molecule with putative membrane receptors [Norman et al., 2001]. Further investigations are required to elucidate the physiological functions of the genomic and non-genomic actions of $1,25(OH)_2D_3$ in the context of whole animals, and these studies are now possible with the aid of VDR knockout mice.

In summary, this study demonstrates unequivocally that $1,25(OH)_2D_3$-induced rapid increases in $[Ca^{2+}]_i$ and PKC activity are neither mediated by, nor dependent upon, a functional nuclear VDR in mouse osteoblasts. Our results support the hypothesis that receptor(s) unrelated to the nuclear VDR mediates the rapid actions of $1,25(OH)_2D_3$.

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