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1,25(OH)$_2$-Vitamin D$_3$ Induces Translocation of the Vitamin D Receptor (VDR) to the Plasma Membrane in Skeletal Muscle Cells

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Abstract 1,25-dihydroxy-vitamin D$_3$ (1,25(OH)$_2$D$_3$), the hormonally active form of vitamin D$_3$, acts through two different mechanisms. In addition to regulating gene expression via the specific intracellular vitamin D receptor (VDR), 1,25(OH)$_2$D$_3$ induces rapid, non-transcriptional responses involving stimulation of transmembrane signal transduction pathways. The activation of second messengers supports the hypothesis that a membrane-bound steroid receptor similar to those that mediate peptide hormone biology exists. Skeletal muscle is a target tissue for 1,25(OH)$_2$D$_3$. Avian embryonic skeletal muscle cells (myoblasts/myotubes) have been shown to respond both genomically and non-genomically to the hormone. The present study provides evidence indicating that short-term treatment (1–10 min) with 1,25(OH)$_2$D$_3$ induces translocation of the VDR from the nuclear to the microsomal fraction in chick myoblasts. This translocation is blocked by colchicine, genistein, or herbimycin, suggesting the involvement of microtubular transport and tyrosine kinase/s in the relocation of the receptor. By isolation of plasma membranes, it was demonstrated that the hormone increases the amounts of VDR specifically in this fraction. These results suggest that the nuclear VDR may be the receptor that mediates the non-genomic effects of 1,25(OH)$_2$D$_3$ in chick myoblasts. J. Cell. Biochem. 86: 128–135, 2002.

Key words: 1,25 (OH)$_2$-vitamin D$_3$; non-genomic effects; muscle cells; vitamin D receptor; translocation; plasma membrane

1,25-dihydroxy-vitamin D$_3$ (1,25(OH)$_2$D$_3$; calcitriol), the hormonally active form of vitamin D$_3$, is an important regulator of mineral homeostasis [Norman et al., 1982; DeLuca and Krisinger, 1990]. Besides this classical function, it has been involved in several physiological responses such as cell growth and differentiation, muscle contractility, and modulation of the immune system [Tanaka et al., 1982; Boland, 1986; Walters, 1992].

1,25(OH)$_2$D$_3$ acts through two different mechanisms [Walters, 1992]. In addition to regulating gene expression via the vitamin D intracellular receptor (VDR) [Minghetti and Norman, 1988; Pelman et al., 1990], like other steroid hormones, 1,25(OH)$_2$D$_3$ induces, rapid, non-transcriptional responses involving the activation of transmembrane signal transduction pathways like growth factors and peptide hormones [De Boland and Nemere, 1992; Nemere and Farach-Carson, 1998].

Skeletal muscle is a target tissue for 1,25(OH)$_2$D$_3$ in which the hormone regulates calcium metabolism and contractility [Boland, 1986]. Avian embryonic skeletal muscle cells (myoblasts/myotubes) have been shown to respond both genomically and non-genomically to the steroid. As an example of the first mechanism, the hormone modulates intracellular

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Ca^{2+} levels stimulating the expression of a Ca^{2+}-binding protein (calbindin-D9K) [Zanello et al., 1995]. On the other hand, $1,25(OH)_2D_3$ induces Ca^{2+} influx by a non-genomic pathway that involves G-protein-mediated activation of phospholipase C [Morelli et al., 1996], thus generating diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), and adenylyl cyclase with the concomitant acute increase in cyclic AMP levels [Vazquez et al., 1995], leading to activation of protein kinase A and C [Vazquez and De Boland, 1996; Vazquez et al., 1997a; Capiati et al., 2000], release of Ca^{2+} from intracellular stores [Vazquez et al., 1997b], and activation of voltage-dependent and store-operated Ca^{2+} channels [Vazquez and De Boland, 1993; Vazquez et al., 1998]. Other non-genomic responses to $1,25(OH)_2D_3$ were described in chick muscle cells such as activation of phospholipases A_2 [De Boland and Boland, 1993] and D [De Boland et al., 1994] and modulation of specific protein kinase C isoforms [Capiati et al., 2001].

The activation of second messengers supports the hypothesis that a membrane-bound receptor similar to those that mediate peptide hormone biology exists. To the present, there is controversial data on this regard. Studies carried out using an antibody raised against a partially purified putative chick intestinal plasma membrane (PM) VDR [Nemere et al., 1994] have reported the presence of such vitamin D binding protein, different from the nuclear VDR, in the PM of chondrocytes [Nemere et al., 1998], enterocytes [Nemere et al., 2000], kidney, and brain [Jia and Nemere, 1999]. Other reports have suggested that annexin II may be the membrane receptor that mediates the rapid actions of $1,25(OH)_2D_3$ in rat osteoblast-like cells ROS 24/1 [Baran et al., 2000]. On the other hand, it has been demonstrated the existence of PM acceptance sites for calcitriol-occupied VDR in ROS 17/2.8 cells [Kim et al., 1996], suggesting that the nuclear VDR, which is known to mediate the genomic actions of $1,25(OH)_2D_3$, may also activate the hormone-induced non-genomic responses.

The present study provides evidence indicating that short-term treatment (1–10 min) with $1,25(OH)_2D_3$ induces translocation of the VDR from the nuclear to the microsomal fraction in chick myoblasts. This translocation is blocked by genistein, herbimycin, or colchicine, suggesting the involvement of tyrosine kinase/s and microtubular transport in the relocation of the receptor. By isolation of PMs, it was demonstrated that the hormone increases the amounts of VDR specifically in this fraction. These results support the hypothesis that the nuclear VDR may be the receptor that mediates the non genomic effects of $1,25(OH)_2D_3$ in muscle cells.

MATERIALS AND METHODS

Materials

$1\alpha,25(OH)_2D_3$ was provided by Dr. P. Weber and Dr. E-M. Gutknecht from Hoffman-La Roche (Basel, Switzerland). Bovine pancreas trypsin, Dulbecco's Modified Eagle's Medium (DMEM), leupeptin, aprotinin, herbimycin A, genistein, daidzein, colchicine, and Immobilon P (polyvinylidene difluoride) membranes were from Sigma Chemical Co. (St. Louis, MO). Western blot chemiluminescence reagents (Renaissance) were provided by New England Nuclear (Chicago, IL). Rabbit polyclonal, highly specific anti-GαS antibody was a gift from Dr. G. Schultz (Pharmakologisches Institut, Freie Universitaet Berlin). Rat monoclonal anti-VDR antibody (clone 9A7) was from Affinity Bioreagents (Golden, CO); anti-lamin B, anti-rat, anti-rabbit, and anti-goat IgG horseradish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Myoblasts were obtained from the breast muscle of 12-day-old white leghorn chick embryos (*Gallus gallus*) by stirring in Earl's balanced salt solution (SSBE) containing 0.1% trypsin during 30 min, essentially as previously described [O'Neill and Stockdale, 1972; Vazquez and De Boland, 1993]. The freed cells were collected by centrifugation and the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution and plated onto glass Petri dishes to remove contaminating fibroblasts and epithelial cells. The unadsorbed, myogenic cells (100,000 cells/cm^2), were placed in plastic dishes (100 mm diameter) and incubated under humidified air with 5% CO_2. The morphological and biochemical characterization of cultures has been previously described [Bellido, 1988; Capiati et al., 1999].

Subcellular Fractionation

Cells were scrapped from the dishes and homogenized in a teflon-glass hand homogen-

izer until greater than 95% of the cells were disrupted (50 strokes) in 10 mM Tris-HCl, pH 7.4, 0.33 M sucrose, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 40 μg/ml leupeptin, 40 μg/ml aprotinin, 1 mM Na_3VO_3, 1 mM NaF. The lysates were subjected to differential centrifugation, first at 200*g* for 10 min to eliminate cell debris, followed by 1,000*g* for 10 min to isolate the nuclear fraction, and 14,300*g* for 20 min to obtain the mitochondrial fraction which was discarded. The supernatant was then centrifuged at 100,000*g* for 1 h for isolation of the cytosolic (supernatant) and microsomal (pellet) fractions. The enrichment of the nuclear specific marker lamin B was assessed by Western blot analysis and the content of PM in the microsomal fraction was determined by adenylyl cyclase assay.

PM Isolation

Purified PM preparations were obtained by a method previously reported with some modifications [Suzuki et al., 1989; Kim et al., 1996]. Muscle cells were collected in TEDK buffer solution (10 mM Tris-HCl, pH 7.4, 0.3 M KCl, 1 mM EDTA, 5 mM dithiothreitol) containing protease inhibitors (1 mM PMSF, 40 μg/ml leupeptin and 40 μg/ml aprotinin) and phosphatase inhibitors (1 mM Na_3VO_3, 1 mM NaF). The cell suspension was homogenized in a teflon-glass hand homogenizer as described above. The homogenized solution was centrifuged at 14,000*g* for 20 min. The supernatant fluid was centrifuged at 100,000*g* for 30 min. The pellet (microsomes) was resuspended in 1.5 ml of 15% sucrose in TEDK and layered on a discontinuous sucrose density gradient composed of 2 ml of 30% sucrose in TEDK layered on to 1.5 ml of 45% sucrose TEDK. This gradient was centrifuged at 76,000*g* for 3 h. The 15–30% (PM fraction) and 30–45% interfaces and the pellet were collected, diluted with TEDK as necessary, and centrifuged at 100,000*g* for 1 h. The resulting pellets were suspended in TEDK plus protease inhibitors. The purity of the PM fraction was assessed by Western blot detection of Gαs (PM specific) and lamin B (nuclei specific), and by determination of glucose-6-phosphatase (endoplasmic reticulum (ER) specific). Gαs represents a more convenient marker to assess the extent of PM purification rather than adenylyl cyclase, as described below for the crude microsomal fraction, since the activity of the enzyme may be affected during subsequent isolation of PMs.

Western Blot Analysis

Protein samples were subjected to SDS–PAGE according to the method of Laemmli [1970]. The separated proteins were electrophoretically transferred to PVDF membranes using a Bio-Rad Semidry Transfer Cell. Non specific sites were blocked with 5% non fat dry milk in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) overnight at 4°C. Membranes were incubated with the respective primary antibody 2 h at room temperature in TBST containing 5% (w/v) non-fat dry milk followed by incubation with the respective horseradish peroxidase-conjugated antibody in TBST 5% non-fat dry milk (antibodies were diluted according to manufacturer's instructions). Bands were visualized by chemiluminescence detection. Images were obtained with a model GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

Adenylyl Cyclase Assay

Adenylyl cyclase was assayed in microsomes to establish the enrichment of this fraction with PMs. Microsomes isolated as described above (see Subcellular Fractionation section) were incubated for 3 min at 30°C in assay solution composed of 50 μM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 50 U/ml creatine phosphokinase, 100 μM 3-isobutyl-1-methylxanthine, 1 mM DTT, 10 mM Tris-HCl, pH 7.4. The reaction was stopped with $HClO_4$, neutralized with $KHCO_3$ (so that the final pH was 5.5–6.0), and the solution was quickly centrifuged at 10,600*g* for 15 min (4°C). Aliquots of the supernatant were taken for cAMP measurements by a radioimmunoassay technique using a commercially available kit [Holmegaard, 1982].

Glucose-6-Phosphatase Assay

Glucose-6-phosphatase was measured by determining the rate of release of inorganic phosphate from glucose-6-phosphate. To begin the reaction, 100 μg of PM protein in a volume of 0.05 ml were added to 0.45 ml of assay mixture containing 22 mM glucose-6-phosphate, 20 mM histidine, and 1 mM EDTA. The reactants were incubated at 37°C for 30 min in a shaking water

bath. The reactions were stopped by adding 2.5 ml of 8% TCA to each tube, centrifuged 15 min at 1,000*g*, and inorganic phosphate was determined in 2 ml aliquots of the supernatant according to the method described by Chen et al. [1956].

Statistical Evaluation

The significance of the results was evaluated by Student's *t*-test, where $P < 0.05$ was considered significant [Snedecor and Cochran, 1967].

RESULTS AND DISCUSSION

VDR levels were determined in chick embryonic muscle cells at different culture times using a monoclonal antibody raised against the nuclear VDR (9A7). A band of ~60 kDa was detected. The receptor levels were low in undifferentiated myoblasts and increased during the differentiation process (Fig. 1). Experiments were then carried out using cells cultured for 4 days in which the VDR is easily detected and cell viability is still high. At this stage of culture development, cells possess biochemical and morphological characteristics of well-differentiated skeletal muscle fibers (myotubes) [Bellido, 1988; Capiati et al., 1999].

The effects of $1,25(OH)_2D_3$ on the subcellular distribution of the muscle VDR were studied. Myotubes were exposed for 5 min to 10^{-9} M $1,25(OH)_2D_3$ and then cytosolic, microsomal, and nuclear fractions were isolated as described under Materials and Methods followed by Western blot analysis for the VDR. As shown in Figure 2A, the nuclear fraction obtained was enriched in the specific nuclear marker lamin B, whereas the microsomal fraction was enriched in adenylyl cyclase activity, indicating that it contained PMs. We have previously ensured that lamin B is absent in the microsomal fraction and adenylyl cyclase activity is absent in the nuclear fraction using a similar cell fractionation protocol [Bellido and Boland, unpublished communications].

Figure 2B shows that in unstimulated cells, the VDR was localized mainly in the nuclear fraction, with very low amounts present in microsomes and almost undetectable levels in cytosol. The hormone increased VDR levels in microsomes ($272.8 \pm 56.6\%$, above control, $P < 0.01$) with a concomitant decrease of the receptor in the nuclear fraction ($-43.7 \pm 8.5\%$, with respect to control, $P < 0.01$). Although the

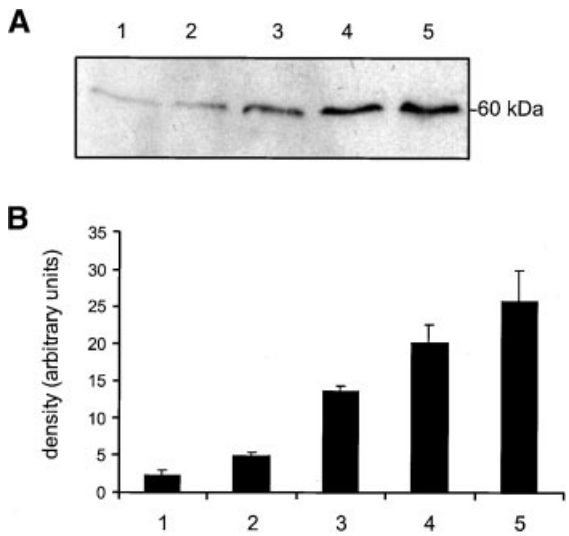

Fig. 1. Expression of the VDR during myogenesis. Whole cell lysate proteins (30 μg) from chick embryonic muscle cells cultured for 1–5 days were subjected to SDS–PAGE and transferred to PDVF membranes. Immunoblotting was performed using anti-VDR antibody. Numbers indicate days of culture. **A**: Representative immunoblot. **B**: Quantitative analysis of blots. Numbers in the x-axis represent days of culture. Results are expressed as density values (arbitrary units) and are the average ± SD of three independent experiments; $P < 0.01$ for 1 vs. 5 days.

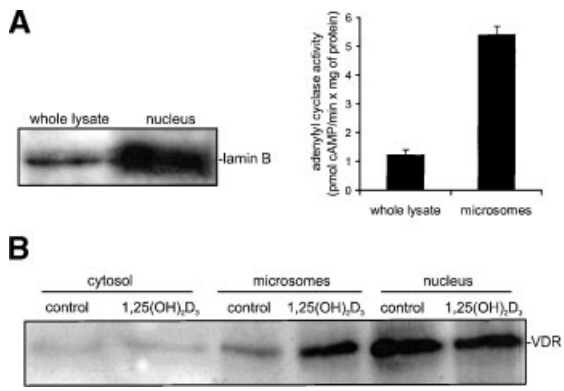

Fig. 2. Effects of $1,25(OH)_2D_3$ on the subcellular distribution of the VDR in muscle cells. **A**: Proteins from chick embryo whole muscle cell lysates, and the nuclear fractions were analyzed for Lamin B content by Western blotting, whereas total lysates and the microsomal fraction were assayed for adenylyl cyclase activity as described in Materials and Methods. **B**: Proteins from cytosol, microsomal, and nuclear fractions (50 μg each) from chick embryonic muscle cells treated during 5 min with 10^{-9} M $1,25(OH)_2D_3$ were subjected to SDS–PAGE and transferred to PVDF membranes. Immunoblotting was performed using anti-VDR antibody. A representative blot of three independent experiments is shown. Quantitative data is given in the text (Results and Discussion).

highest effect of $1,25(OH)_2D_3$ on VDR translocation to microsomes was observed after 5 min of treatment, it also occurred after 1 or 10 min (87.4 ± 2.8 and $80.3 \pm 30.6\%$ above controls, $P < 0.001$ and $P < 0.05$, respectively). The possibility that the increase of VDR in microsomes reflects preferential VDR stabilization in this fraction caused by the rapid exposure of muscle cells to $1,25(OH)_2D_3$ may be excluded. Western blot analysis revealed no differences in total cellular VDR before and after hormone treatment (data not shown).

Previous studies reported that after long-term treatment (hours) with the hormone, the $1,25(OH)_2D_3$–VDR complex is translocated to the nucleus via microtubular transport in monocytes [Kamimura et al., 1995]. Treatment of myotubes with the inhibitor of microtubule assembly colchicine was carried out to determine whether VDR translocation to the microsomal fraction in response to the hormone involved the same mechanism. Colchicine (1 and 50 μM) blocked VDR translocation in a dose-dependent manner (Fig. 3). Treatment with colchicine alone did not affect basal levels of the receptor in microsomes. These data indicates that the VDR is transported from the nucleus to membranes via microtubules in myotubes.

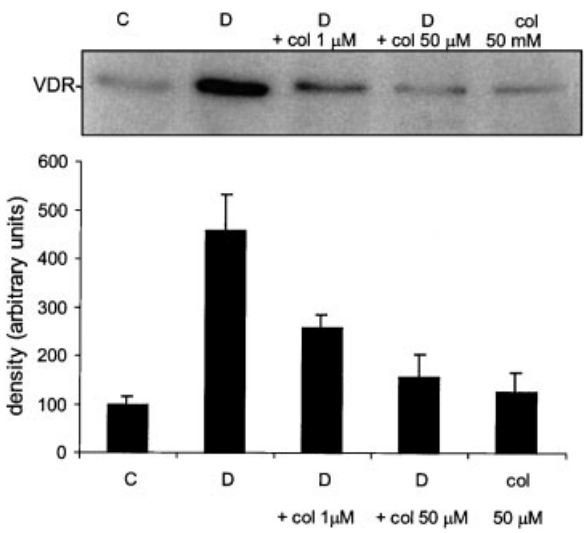

Fig. 3. Effects of colchicine on $1,25(OH)_2D_3$-dependent translocation of the VDR in muscle cells. Chick muscle cells were treated for 5 min with 10^{-9} M $1,25(OH)_2D_3$ in the absence and presence of 1 or 50 μM colchicine (col), which was added 5 min prior to hormone treatment. The cells were then homogenized followed by isolation of subcellular fractions and Western blot analysis of the VDR. **Top**: Representative immunoblots obtained with microsomal fractions. **Bottom**: Quantitative analysis of blots; results are expressed as density values (arbitrary units) and are the average ± SD of three independent experiments. C, control; D, $1,25(OH)_2D_3$. $P < 0.05$ for C vs. D; D vs. D + col 1 μM and D vs. D + col 50 μM.

To obtain more information about the mechanism of translocation, the involvement of tyrosine kinase/s was addressed. Treatment of myotubes with the tyrosine phosphorylation inhibitors genistein (100 μM) or herbimycin (5 μM) blocked VDR translocation to the microsomal fraction (Fig. 4A,B). Daidzein, an inactive analog of genistein, had no effect. Treatment with genistein or herbimycin alone did not affect basal levels of the receptor in microsomes. These results indicate that tyrosine kinase(s) are involved in the signaling mechanism, which mediates VDR translocation itself or association of the receptor to other proteins located in the membranes. In accordance with the latter interpretation, it has been shown that $1,25(OH)_2D_3$ increases tyrosine phosphorylation of the VDR and its interaction with c-Src in chick myotubes [Buitrago et al., 2000]. Through myristoylation, c-Src is associated to the PM and its SH2 domains might interact with tyrosine phosphorylated residues on the VDR, locating the receptor in the PM.

The data so far shown were obtained analyzing the microsomal fraction, which contains not only PMs, but other components of the endomembrane system, such as ER. In order to determine whether the VDR translocates specifically to the PM, this membrane system was purified by sucrose density gradient centrifugation of microsomes isolated from myotubes treated for 5 min with 10^{-9} M $1,25(OH)_2D_3$ followed by Western blot analysis for the VDR. This PM fraction was enriched in Gαs protein, a selective marker for PM (Fig. 5A), with no detectable contamination of nucleus fragments, as indicated by the absence of lamin B (Fig. 5B). Only traces of ER fragments, determined by glucose-6-phosphatase assay, were detected in the purified PM preparation (not shown). This expected contamination, inherent to the isolation method, is similar to that observed in other cell systems [Kim et al., 1996]. As shown in Figure 5C, $1,25(OH)_2D_3$ increased the amounts of VDR associated to the PM ($420.7 \pm 98.3\%$, above control, $P<0.01$). Of interest, by densitometric quantification of VDR Western blots, taking into account the amounts of PM proteins relative to those of whole cell lysate proteins, it could be estimated that the concentration of VDR associated to the PM after $1,25(OH)_2D_3$

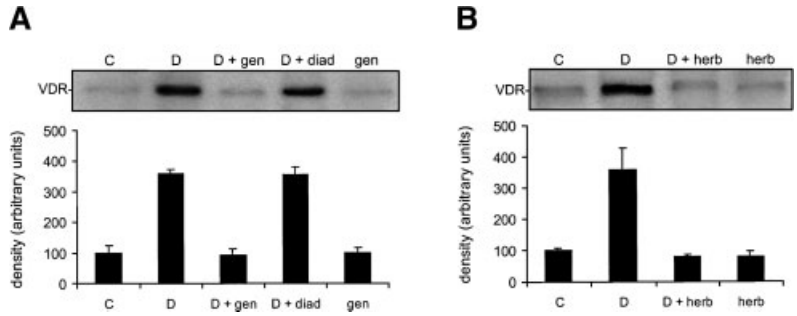

Fig. 4. Effects of tyrosine phosphorylation inhibitors on $1,25(OH)_2D_3$-dependent translocation of the VDR in muscle cells. Chick muscle cells were treated for 5 min with 10^{-9} M $1,25(OH)_2D_3$ in the absence and presence of 100 μM genistein or 100 μM daidzein (**A**) or 5 μM herbimycin A (**B**), which were added 5 min prior to hormone treatment. The cells were then homogenized followed by isolation of subcellular fractions and Western blot analysis of the VDR. **Top**: Representative immunoblots obtained with microsomal fractions. **Bottom**: Quantitative analysis of blots; results are expressed as density values (arbitrary units) and are the average ± SD of three independent experiments. C, control; D, $1,25(OH)_2D_3$; gen, genisten; daid, daidzein; herb, herbimycin A. A: $P < 0.001$ for C vs. D; C vs. D + daid and D vs. D + gen. B: $P < 0.01$ for C vs. D and D vs. D + herb.

treatment represents ~30% of the total receptor content.

Traces of VDR were detected in the other microsomal subfractions (30–45% interface and pellet), but no translocation of the receptor to these fractions was observed (Fig. 5C); therefore, the increase of VDR levels in the PM fraction was not due to the presence of contaminant fragments from other microsomal components such as ER.

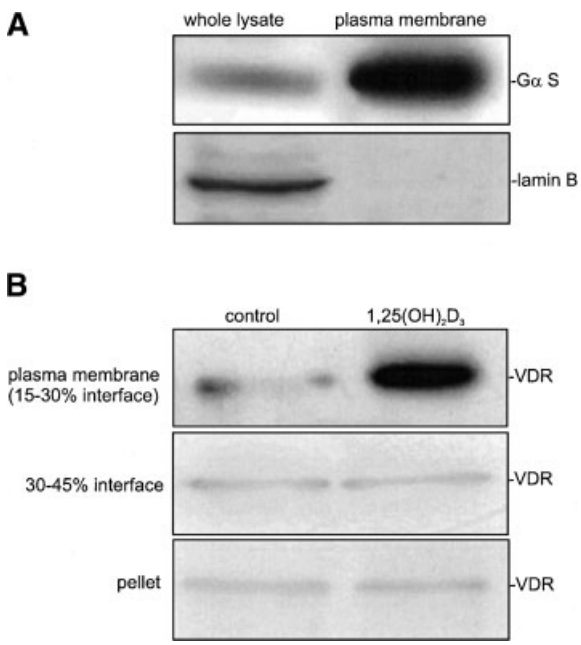

Fig. 5. Effects of $1,25(OH)_2D_3$ on PM associated VDR levels in muscle cells. **A**: Characterization of the PM fraction. Proteins from chick embryo whole muscle cell lysates and PM fraction were subjected to Western blot analysis for Gαs protein and lamin B. **B**: Muscle cells were treated for 5 min with 10^{-9} M $1,25(OH)_2D_3$. The cells were then homogenized followed by isolation of PM fraction (15–30% interface), 30–45% interface and pellet as described in Materials and Methods and Western blot analysis of the VDR. Quantitative data are given in the text.

In conclusion, the results obtained in this study indicate that short-term treatment with $1,25(OH)_2D_3$ induces a rapid movement of the VDR from the nucleus to the PM in chick myotubes via microtubular transport and that tyrosine kinase(s) participate in the translocation process and/or association of the VDR to membrane located proteins. More studies are needed to determine whether it is the ligand-receptor complex or the VDR alone, which translocates from the nucleus to the PM in response to the hormone in chick muscle cells. Interactions of the nuclear $1,25(OH)_2D_3$–VDR complex with plasma membrane acceptance sites have been demonstrated in ROS 17/2.8 cells [Kim et al., 1996].

Our data supports the hypothesis that the classic nuclear VDR may be the receptor that mediates, at least in part, the non-genomic effects of $1,25(OH)_2D_3$ in chick myotubes. In agreement with this interpretation, by applying antisense technology, it has been shown that the VDR mediates rapid hormone-induced changes in muscle cell protein tyrosine phosphorylation [Buitrago et al., 2001]. Congruent with the present evidence on cell membrane localization of the classic VDR, a $1,25(OH)_2D_3$-dependent association between the VDR and the store-operated calcium (SOC) channel forming TRP protein has been shown in cultured muscle cells [Santillan et al., 2000]. Moreover, prior transfection of these cells with an anti-

VDR antisense oligonucleotide suppresses hormone stimulation of Ca^{2+} influx through SOC channels [Santillan and Boland, unpublished communications]. Other studies showed the presence of a $1,25(OH)_2D_3$ binding protein of 65–66 kDa different from the nuclear VDR in various cell types, which has been functionally related to transcaltachia and rapid activation of PKC [Nemere et al., 1994, 1998, 2000; Jia and Nemere, 1999]. Alternatively, it has been suggested that annexin II may be the membrane receptor that mediates $1,25(OH)_2D_3$-induced rapid increases in cytosolic Ca^{2+} in rat osteoblast-like cells ROS 24/1, which have very few or undetectable VDR [Baran et al., 2000]. It is then possible that the great variety of non-genomic effects exerted by $1,25(OH)_2D_3$ in its target cells can be mediated by different receptors/binding proteins.

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