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FAST TRACKS

The Classic Receptor for 1α,25-dihydroxy Vitamin D3 is Required for Non-Genomic Actions of 1α,25-Dihydroxy Vitamin D3 in Osteosarcoma Cells

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Abstract 1α,25-dihydroxy vitamin D3 has a major role in the regulation of the bone metabolism as it promotes the expression of key bone-related proteins in osteoblastic cells. In recent years it has become increasingly evident that in addition to its well-established genomic actions, 1α,25-dihydroxy vitamin D3 induces non-genomic responses by acting through a specific plasma membrane-associated receptor. Results from several groups suggest that the classical nuclear 1α,25-dihydroxy vitamin D3 receptor (VDR) is also responsible for these non-genomic actions of 1α,25-dihydroxy vitamin D3. Here, we have used siRNA to suppress the expression of VDR in osteoblastic cells and assessed the role of VDR in the non-genomic response to 1α,25-dihydroxy vitamin D3. We report that expression of the classic VDR in osteoblasts is required to generate a rapid 1α,25-dihydroxy vitamin D3-mediated increase in the intracellular Ca^{2+} concentration, a hallmark of the non-genomic actions of 1α,25-dihydroxy vitamin D3 in these cells. J. Cell. Biochem. 99: 995–1000, 2006.

Key words: vitamin D3 receptor; non-genomic actions of vitamin D3; osteoblastic cells

Bone cells are one of the main targets for 1α,25-dihydroxy vitamin D3, the most active metabolite of vitamin D [Christakos et al., 2003]. In osteoblastic cells 1α,25-dihydroxy vitamin D3 promotes synthesis of extracellular matrix proteins and the subsequent mineralization process [Christakos et al., 2003]. In recent years it has become increasingly accepted that steroid hormones in general, and 1α,25-dihydroxy vitamin D3 in particular, exert rapid non-genomic actions [Norman et al., 2004]. These rapid responses to 1α,25-dihydroxy vitamin D3 are initiated at the cell membrane level and occur within a range of minutes. Among the non-genomic actions of 1α,25-dihydroxy vitamin D3 are the opening of L-type Ca^{2+} channels in osteoblasts which results in a rapid increase of intracellular Ca^{2+} [Zanello and Norman, 1996], activation of PKC [Boyan et al., 1994; Nemere et al., 1998; Schwartz et al., 2002], activation of phospholipase C [Boyan et al., 1998], and activation of MAPK [Buitrago et al., 2001; Boland et al., 2002].

Early reports postulated a specific membrane-associated receptor as responsible for the rapid non-genomic responses elicited by 1α,25-dihydroxy vitamin D3 [Nemere et al., 1998; Norman et al., 2002; Schwartz et al., 2002]. However, recent results suggest that the classic nuclear 1α,25-dihydroxy vitamin D3 receptor (VDR), which performs the genomic actions of this hormone, is also responsible for the non-genomic actions [Zanello and Norman, 2004a]. Thus, it has been found that the classical VDR is associated with the caveolae in the

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plasma membrane of several cell types, including osteoblasts [Huhtakangas et al., 2004], where it can bind 1α,25-dihydroxy vitamin D3. Also the VDR has been found to translocate to the membrane of skeletal muscle cells in response to stimulation with 1α,25-dihydroxy vitamin D3 [Capiati et al., 2002]. More interestingly, Norman and colleagues have recently shown that 1α,25-dihydroxy vitamin D3-mediated rapid modulation of Ca^{2+} and Cl^{-} channels in mouse calvarial osteoblasts occurs only in the presence of a functional classic VDR. Thus, they reported that osteoblasts isolated from VDR knock out mice, lack this 1α,25-dihydroxy vitamin D3-induced rapid non-genomic response [Zanello and Norman, 2004b]. However, there is also a requirement to perform these analyses in osteoblastic cells that normally express VDR and which can be transiently deprived of it.

Therefore, we have developed an siRNA assay that specifically targets the rat VDR in osteoblastic ROS 17/2.8 cells and demonstrated its key role in the non-genomic actions of 1α,25-dihydroxy vitamin D3. We find that when the expression of VDR in these cells is suppressed, the 1α,25-dihydroxy vitamin D3-mediated increase in intracellular Ca^{2+} levels is abolished.

MATERIALS AND METHODS

Cell Culture

ROS 17/2.8 osteoblastic cells [rat osteosarcoma-derived cells, Majeska et al., 1980] were culture onto glass cover slips for the intracellular calcium measurements or in six-well dishes for immunodetection (Western blot) and RT-PCR analysis. Cells were maintained in F12 medium (Gibco-BRL, Carlsbad, CA) containing 5% FBS (Hyclone, Logan, UTA) under humidified air (5% CO_2) at 37°C, as described earlier.

Intracellular Calcium Measurements

Calcium intracellular changes were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2/AM (cell permanent acetoxymethyl ester, Molecular Probes, Eugene, OR). Cells grown onto glass cover slips were incubated in PBS containing 5 μM of the penta-acetoxymethylester derivate, in darkness during 30 min at 37°C. Unloaded dye was washed out and the cells maintained in a saline solution containing 150 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (30 mM sucrose was added to obtain an osmolarity of 320). For the fluorescence measurements, the cover slips containing dye-loaded cells were mounted on the stage of an inverted microscope (Nikon Eclipse TE-300, Melville, NY) equipped with a system of exchange excitation filters (340/380 nm) and a digital ratio imaging system Axon Imaging Workbench (Axon Instruments, CA). A digital CCD camera using a 510 nm filter collected the single cell emitted fluorescence. Variations in intracellular calcium levels were registered during 5 min at room temperature (18°C). It was established that measurements at 10 s intervals provided an accurate result, minimizing the effects of photo bleaching. Resting levels corresponding to changes in intracellular Ca^{2+} in unstimulated cells were monitored during 300 s. The extent to which intracellular Ca^{2+} concentrations were raised in response to 1α, $25(OH)_2D_3$ or the vehicle ethanol, was determined using the Tsien formula [Grynkiewicz et al., 1985], calculating the percentage of increase of the F_{340}/F_{380} ratio with respect to the value at resting state. The estimated Kd value for the Fura-2/Ca^{2+} binding was 287 nM. The Origin 6.0 (Microcal, Inc., CA) software was used to generate the curves that represent intracellular Ca^{2+} variations in individual cells ($n = 10$ in each experiment).

siRNA

To silence endogenous VDR protein expression in ROS 17/2.8 cells, we used the Silencer siRNA Construction Kit (Ambion, Austin, TX) following the manufacturer's recommendations. A 19-nucleotides probe directed against the sequence spanning nucleotides 461–481 (5′-AAGCTATCTGAAGAACAACAG-3′) of the rat VDR gene (hinge domain, Pub Med accession number NM_017058) was designed and transfected into ROS 17/2.8 cells by the Oligofectamine Reagent (Invitrogen, Carlsbad, CA). Effective silencing of the VDR expression was assessed by Western blotting and RT-PCR (see below).

Western Blot Analysis

Whole cell extracts (WCE) were obtained by adding 150 μl of lysis buffer (2% SDS, 2 M Urea, 100 mM Tris-HCl pH 6.8, 0.5 mM PMSF, and a protease inhibitors cocktail) to ROS 17/2.8 cells

grown on 35 mm diameter wells. The mix was incubated 30 min at 4°C, recovered by pipetting and centrifuged 5 min, 4°C at maximum speed in a microfuge. The protein extracts were collected and then fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then blotted using either anti VDR (C-20) or anti β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The detection was performed using Western Lightning Chemiluminiscence Reagent Plus (Perkin Elmer Life Science, Boston, MA). The quantification of the results was carried out by using a Molecular Imager System (FX, Bio-Rad).

RNA Isolation and RT-PCR

Total RNA was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA pellet was suspended in Diethyl-pyrocarboante (DEPC, Sigma-Aldrich, Saint Louis, MO) -treated water supplemented with 0.2 U/μl of RNAsin (Promega, Madison, WI) quantified in a spectrophotometer (SmartSpect 3000, Bio-Rad) and stored at −80°C until use. For the RT reaction, 1 μg of total ARN were incubated with an antisense VDR oligonucleotide [Liel et al., 1999] or an antisense β-actin oligonucleotide (5′-CAAACATGATCTGGGTCATCTTCT-3′) in a 10 μl final volume, at 70°C for 10 min and at 4°C for 5 min, respectively. Ten microliters of RT mix were then added [100 U MMLRT (Promega), MMLRT 1X buffer (Promega), 14 U of RNAsin (Promega) and 0.5 mM of each dNTP (Invitrogen)] and incubated at 42°C for 1 h. The reaction was stopped by placing the mix on ice. PCR was carried out by adding 2 μl of cDNA (previously diluted 5X in DEPC-treated water) into 50 μl final volume of the PCR mix (0.2 mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol sense and antisense primers, Taq Pol buffer 1X (Invitrogen) and, 2.5 U Taq Pol enzyme (Invitrogen)). The annealing temperature used was 55°C.

RESULTS AND DISCUSSION

To evaluate the contribution of the classical VDR in the non-genomic actions of 1α,25-dihydroxy vitamin D3 in osteoblastic cells, we developed a protocol to transiently inhibit the expression of VDR in rat-derived ROS 17/2.8 osteosarcoma cells using the siRNA method [Dykxhoorn et al., 2003]. To target the VDR mRNA we generated siRNA directed against the region encoding for the hinge domain of the VDR protein (nt 461–481). This domain represents the most divergent motif among the members of the nuclear steroid receptors family [Olefsky, 2001] and therefore increases the specificity of our inhibitory RNA.

As shown in Figure 1, we found that 24 h after transfecting the cells with the siRNA there is an 80% reduction in both VDR message (Fig. 1B) and VDR protein (Fig. 1D) levels. This inhibition is reversible as 48 h later, the VDR protein levels raise and the VDR-mediated functions are restored (data not shown). The inhibition is also specific as the ROS 17/2.8 cells subjected to the same transfection procedure but without including the siRNA molecules, do not exhibit a detectable reduction in the VDR mRNA or protein concentration (Fig. 1A, compare lanes 1 and 2). Similarly, neither β-actin mRNA nor

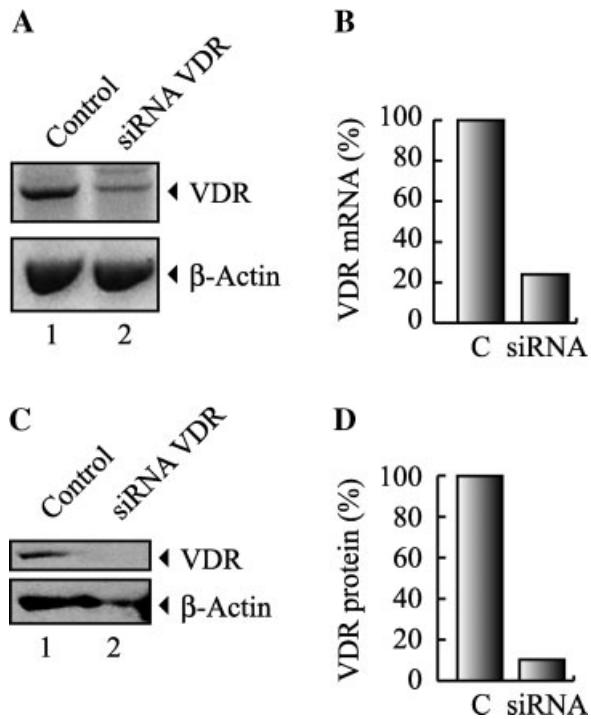

Fig. 1. Expression of VDR in ROS 17/2.8 cells is suppressed by siRNA. ROS 17/2.8 cell cultures were transfected with a siRNA that targets the VDR expression. The reduction in VDR expression was determined after 24 h by both RT-PCR (**A** and **B**) and Western blot (**C** and **D**). Control lane corresponds to ROS 17/2.8 cells transfected without the specific siRNA. Expression of β-actin mRNA (A and B) and protein (C and D) were used as a control for siRNA specificity. C and D correspond to the quantification of the results shown in A and B.

β-actin protein levels are affected when the expression of VDR is suppressed (Fig. 1A, C, lower panels).

The opening of L-type Ca^{2+} channels and the rapid increase in intracellular Ca^{2+} concentration represents one of the hallmarks of the non-genomic actions of 1α,25-dihydroxy vitamin D3 in osteoblasts [Zanello and Norman, 2004a]. It has been established that this response is achieved after a short (3–5 min) incubation with nanomolar concentrations of 1α,25-dihydroxy vitamin D3 [Caffrey and Farach-Carson, 1989; Yukihiro et al., 1994; Baldi et al., 2002; Zanello and Norman, 2004a]. In agreement with these previous studies, we determined that treatment of wild-type ROS 17/2.8 cells with 10 nM 1α,25-dihydroxy vitamin D3 is sufficient to produce a rapid and sustained increase in intracellular Ca^{2+} levels (up to 180 nM) within 3 min of incubation (Fig. 2A).

Previous reports have also indicated that incubation of several cell types with ethanol produces a rapid elevation of intracellular Ca^{2+}. This increase has been attributed to both release from intracellular stores and stimulation of voltage-gated channels [Belia et al., 1995; Yang et al., 1999]. Because ethanol is used as a vehicle to dissolve the 1α,25-dihydroxy vitamin D3 added to the cells, we evaluated whether the ROS 17/2.8 cells also respond to pharmacological concentrations of ethanol under our experimental conditions. It was determined that a short treatment of these osteoblastic cells with 10 mM ethanol (or higher concentrations, not shown) results in a rapid elevation of the intracellular Ca^{2+} concentration (Fig. 2B). However, this response is not observed at lower concentrations of ethanol (3 mM or less), which are equivalent to those present in the media (1 mM or less) after 1α,

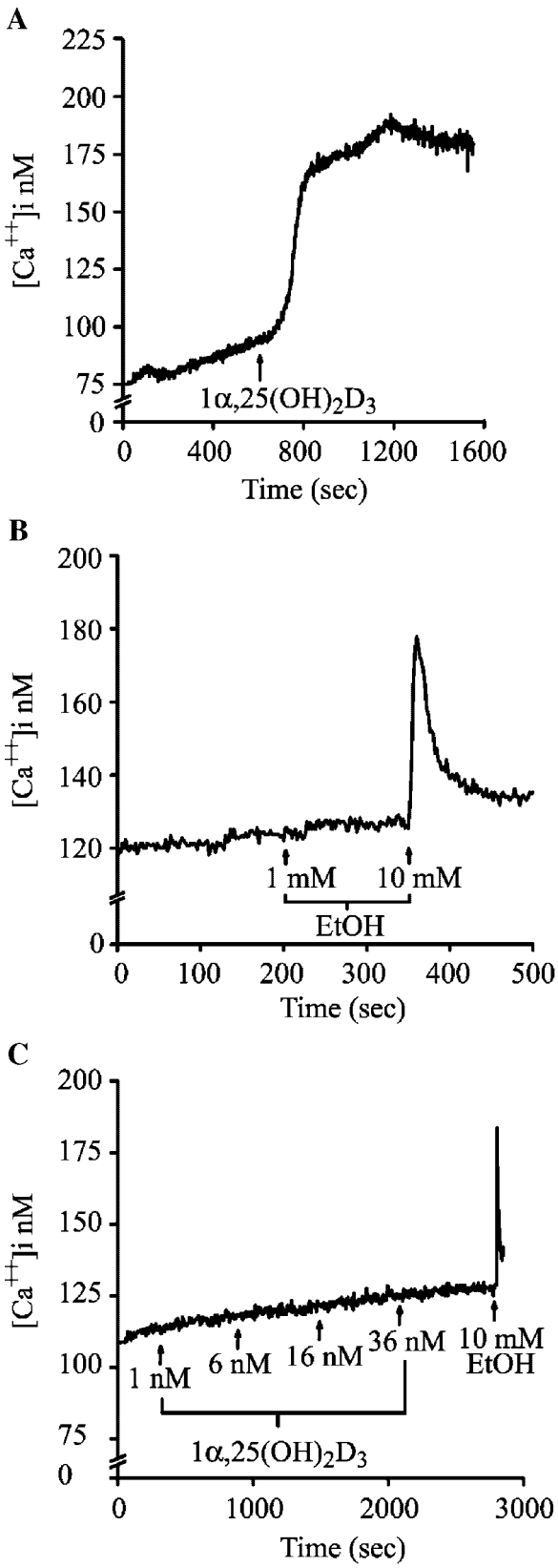

Fig. 2. Inhibition of the VDR expression in ROS 17/2.8 cells results in inability to elicit non-genomic responses to 1α,25-dihydroxy vitamin D3. ROS 17/2.8 cell cultures were exposed to 10 nM 1α,25-dihydroxy vitamin D3 (**A**) or 10 mM ethanol (**B**) and the increase of intracellular Ca^{2+} concentration measured at the single cell level as described in Materials and Methods. After exposure to either 1α,25 dihydroxy vitamin D3 or ethanol, the cells raise their intracellular Ca^{2+} levels from 100 to 180 nM. **C**: ROS 17/2.8 cells transfected with the siRNA that targets VDR were exposed to increasing concentrations of 1α,25-dihydroxy vitamin D3 or 10 mM ethanol and their ability to respond by raising the intracellular Ca^{2+} levels. The values exhibited in graphs correspond to one registered cell each, representing the average response observed within groups of 10 cells (n = 10), that were evaluated in several independent experiments.

25-dihydroxy vitamin D3 is added to the cells (data not shown). In addition, it appears that the response to ethanol is of a different nature than that generated by 1α,25-dihydroxy vitamin D3, as the ethanol-mediated increase in Ca^{2+} concentration is markedly transient (compare Fig. 2A, B). Taken together, these results indicate that under our experimental conditions ROS 17/2.8 cells exhibit a rapid, sustained and saturable non-genomic response to 1α,25-dihydroxy vitamin D3.

We next evaluated whether the VDR-less ROS 17/2.8 cells lose or retain the ability to elicit non-genomic responses to 1α,25-dihydroxy vitamin D3. As shown in Figure 2C, we determined that the osteoblastic cells that lack VDR do not exhibit an increase in intracellular Ca^{2+} in response to increasing concentrations of 1α,25-dihydroxy vitamin D3. Therefore, these cells do not elicit this 1α,25-dihydroxy vitamin D3-mediated non-genomic action. Interestingly, these cells still retain their ability to respond to ethanol (10 mM) by elevating their intracellular Ca^{2+} levels (Fig. 2C). This result indicates that the ethanol-induced effect is independent of VDR and further suggests that involves a different mechanism.

In conclusion, here we demonstrate that osteoblastic cells that normally express VDR, require this protein to generate a rapid increase in intracellular Ca^{2+} concentration, a hallmark among the non-genomic responses reported for 1α,25-dihydroxy vitamin D3 in this type of cells [Caffrey and Farach-Carson, 1989; Yukihiro et al., 1994; Baldi et al., 2002; Zanello and Norman, 2004a]. Our results are in agreement with previous reports indicating that osteoblastic cells isolated from VDR knock out mice do not exhibit rapid modulation of Ca^{2+} and Cl^{-} channels in response to 1α,25-dihydroxy vitamin D3 [Zanello and Norman, 2004b]. Taken together, these results strongly indicate that the rapid non-genomic actions initiated by 1α,25-dihydroxy vitamin D3 at the plasma membrane are mediated by the classical VDR molecule.

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