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Induction of apoptosis by 1,25-dihydroxyvitamin D_3 in MCF-7 Vitamin D_3-resistant variant can be sensitized by TPA☆

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Abstract

Vitamin D_3 compounds offer an alternative approach to anti-hormonal therapies for human breast cancer. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) acts through the nuclear Vitamin D_3 receptor (VDR), a phosphoprotein and ligand-dependent transcription factor. Our lab has shown that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species (ROS). TPA, a protein kinase C (PKC) activator, does not induce cytochrome *c* release or Bax translocation, thus demonstrating that it has no effect on mitochondria and apoptosis on its own. However, when the MCF-7^{D_3Res} cells (a Vitamin D_3-resistant variant) are treated with 1,25-$(OH)_2D_3$ in the presence of TPA, the cells displayed apoptotic morphology and redistribution of both cytochrome *c* and Bax. TPA pretreatment greatly enhances 1,25-$(OH)_2D_3$ stimulated 24-hydroxylase luciferase activity and VDR protein expression, although transactivation is lower in the MCF-7^{D_3Res} cells compared to the parental cell line. The observation that the phorbol ester TPA sensitizes the Vitamin D_3-resistant variant to the effects of 1,25-$(OH)_2D_3$ suggests an important role for phosphorylation in dictating sensitivity to Vitamin D_3-mediated apoptosis. This study demonstrates that the effects of 1,25-$(OH)_2D_3$ on mitochondrial disruption might be sensitized through activators of PKC.

Keywords: Vitamin D_3; Apoptosis; TPA; Mitochondria

1. Introduction

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of Vitamin D_3, is not only a powerful regulator of calcium homeostasis, but is a steroid hormone with important roles in cell growth and differentiation. 1,25-$(OH)_2D_3$ is a potent negative growth regulator of breast cancer cells both in vitro and in vivo. A variety of synthetic Vitamin D_3 analogs that induce breast tumor regression in animals are now undergoing clinical trials in human patients [1,2]. Our lab has shown that inhibition of breast cancer cell growth in response to 1,25-$(OH)_2D_3$ involves activation of apoptosis [3,4]. 1,25-$(OH)_2D_3$ acts through the nuclear Vitamin D_3 receptor (VDR), a ligand-dependent transcription factor with major phosphorylation sites occurring on serine residues. Previous studies have shown that the VDR can be phosphorylated by protein kinase C (PKC), protein kinase A (PKA), and casein kinase II; however, the effects of phosphorylation on receptor expression or activation are poorly understood [5–7].

One goal of the present study was to expand our findings on the specific intracellular events involved in 1,25-$(OH)_2D_3$-mediated apoptosis, in particular, the role of mitochondria in commitment of cells to apoptosis. Translocation of Bax from cytosol to mitochondria, increased permeability of the outer mitochondrial membrane, cytochrome *c* release and generation of reactive oxygen species (ROS) may initiate disruption of mitochondrial function [8,9]. Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can block these mitochondrial events and prevent apoptosis. Events downstream of mitochondrial disruption are characterized by the activation of caspases and nucleases that lead to the ultimate destruction of the cell.

We have previously shown that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function, and that these mitochondrial effects are not blocked by the cell permeable caspase inhibitor zVAD.fmk [10]. Although caspase inhibition blocks 1,25-$(OH)_2D_3$-mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-$(OH)_2D_3$-mediated cell death is caspase-independent.

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Moreover, Bax translocation and mitochondrial disruption do not occur after 1,25-$(OH)_2D_3$ treatment of the Vitamin D_3-resistant variant, MCF-7^{D_3Res} cells [10].

We have reported that the phorbol ester TPA (a PKC activator) can potentiate the effects of 1,25-$(OH)_2D_3$ on induction of apoptosis, and more significantly, that TPA can partially sensitize the MCF-7^{D_3Res} cells to 1,25-$(OH)_2D_3$-mediated apoptosis [4]. We thus hypothesized that TPA-mediated phosphorylation pathways might enhance 1,25-$(OH)_2D_3$ signaling and mitochondrial disruption. Hence, a second goal of this study was to assess whether activators of PKC affect VDR expression, specific DNA binding, and/or transactivation in sensitive and resistant MCF-7 cells in relation to apoptosis following treatment with 1,25-$(OH)_2D_3$ or TPA.

In the present study, we provide additional evidence for the involvement of the Bcl-2 family of apoptotic regulators in 1,25-$(OH)_2D_3$-mediated apoptosis. Furthermore, we report that TPA alone does not alter mitochondrial function but is able to augment the effects of 1,25-$(OH)_2D_3$ on mitochondria, enhance VDR protein expression and transactivation, and most importantly, potentiate apoptosis in MCF-7 cells and the Vitamin D_3-resistant variant.

2. Materials and methods

2.1. Cells and cell culture

MCF-7 cells were used to generate the 1,25-$(OH)_2D_3$-resistant variant (MCF-7^{D_3Res}) that has been previously described [11]. Both cell lines were cultured in α-MEM medium (Life Technologies, Inc., Gaithersburg, MD) containing 25 mM HEPES and 5% FBS (Life Technologies). Stock cultures of MCF-7^{D_3Res} cells were routinely grown in medium containing 100 nM 1,25-$(OH)_2D_3$ (kindly provided by LEO Pharmaceuticals, Ballerup, Denmark). For experiments, parental MCF-7 and MCF-7^{D_3Res} cells were plated in α-MEM containing 5% FBS plus antibiotics, and treatments with 1,25-$(OH)_2D_3$, TPA, or ethanol vehicle were initiated 2 days after plating. In long-term experiments, cells were re-fed with fresh medium containing treatments 2 or 3 days later.

2.2. Cell sorting and subcellular fractionation

A suspension of (1–2) × 10^7 MCF-7 cells was incubated with 10 μg/ml annexin V-FITC (PharMingen, San Diego, CA) in binding buffer (10 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min at 37 °C and sorted into apoptotic (annexin positive) and viable (annexin negative) populations using a Coulter Altra Cell Sorter (Coulter Corp., Miami, FL). Cytosolic and membrane fractions were prepared by lysing the collected cell populations in hypotonic lysis buffer (10 mM HEPES, pH 7.5, 10 mM NaCl containing protease and phosphatase inhibitors) for 30 min on ice, followed by nine cycles of freezing in liquid nitrogen and thawing at 37 °C. The crude lysate was centrifuged (17,000 rpm, 15 min, 4 °C) to obtain a cytosolic (supernatant) and membrane (pellet) fraction.

2.3. Immunoblot analysis

Cytosolic or membrane fractions derived from equal numbers of cells, were solubilized in Laemmli buffer, separated by SDS-PAGE gel, and transferred to nitrocellulose. Blots were incubated with Bax rabbit polyclonal (13666E; PharMingen) or Bcl-2 mouse monoclonal antibodies (Dako, Carpinteria, CA) diluted 1:500 or 1:100 in PBS/5% skim milk, respectively. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:5000 in PBS/5% skim milk/0.1% Tween 20 and autoradiography with enhanced chemiluminescence (Pierce). For VDR, proteins from high salt nuclear extracts, prepared in the presence of phosphatase inhibitors, were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with a monoclonal antibody directed against the VDR (clone 9A7γ; Lab Vision Corporation, Fremont, CA) diluted 1:50 in PBS/5% skim milk followed by horseradish peroxidase-conjugated anti-rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and chemiluminescent detection.

2.4. Immunocytochemistry

MCF-7 or MCF-7^{D_3Res} cells were grown on two-well Lab-Tek II chamber slides (Fisher Scientific) and treated 2 days after plating with ethanol, 1 nM TPA, 100 nM 1,25-$(OH)_2D_3$, or both for 96 h. Cells were fixed in 4% formaldehyde, permeabilized in ice-cold methanol and blocked overnight with PBS/1% BSA. Slides were incubated with cytochrome *c* mouse monoclonal antibody (6H2.B4; PharMingen) and Bax rabbit polyclonal (Upstate Biotechnology, Inc., Lake Placid, NY) diluted 1:20 and 1:100 in blocking buffer, respectively, for 2 h at 37 °C. Slides were washed 3 × 5 min with PBS followed by incubation for 1 h at room temperature with anti-mouse secondary antibody conjugated to ALEXA-488 (fluorescein; Molecular Probes, Eugene, OR) and anti-rabbit secondary conjugated to ALEXA-568 (rhodamine; Molecular Probes) diluted 1:100 and 1:200 in blocking buffer, respectively. Slides were washed 3 × 5 min with PBS, incubated for 15 min at room temperature with 1 μg/ml Hoechst 33258 (Sigma), washed 5 × 5 min with PBS, rinsed with dH_20, and mounted with antifade reagent. Fluorescence was detected using Olympus AX70 microscope equipped with a Spot RT digital camera.

2.5. Flow cytometry

Adherent and floating cells were harvested by trypsinization and pelleted. For analysis of mitochondrial membrane

potential or ROS production, cell suspensions (1×10^6 cells) were incubated in the presence of 1 μM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) or 4 μM hydroethidine (HE, Molecular Probes) as previously described [10]. Analysis of TMRE red fluorescence or conversion of HE to ethidium was analyzed by flow cytometry (further described below).

For detection of phosphatidylserine externalization, 1×10^6 cells were incubated with 10 μg/ml annexin V-FITC (PharMingen) and 5 μg/ml propidium iodide (PI) in binding buffer for 15 min at 37 °C. The cells were washed twice in binding buffer, fixed in 2% formaldehyde in PBS for 15 min on ice, and then washed two more times in PBS/0.2% PBS. The pellet was resuspended in PBS/0.2%BSA and analyzed by flow cytometry. There were less than 1% PI^+ cells in the population and these were excluded from analysis. Observations were confirmed by fluorescence microscopy in which cells grown on chamber slides were incubated with annexin V-FITC and Hoechst 33258.

Analysis was performed on an Epics XL Flow Cytometer (Coulter Corp., Miami, FL) equipped with an argon laser. TMRE and HE were analyzed on FL3 using a 620 nm band pass filter. For PS externalization, annexin V-FITC was analyzed on FL1 and PI was analyzed on FL2 (580 nm band pass filter) using software color compensation. Data was analyzed using MULTIPLUS AV analysis software (Phoenix Flow Systems).

2.6. In vitro alkaline phosphatase reactions

Alkaline phosphatase reactions were performed on nuclear extracts derived from parental MCF-7 and MCF-7^{D_3Res} cells treated with 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA, or ethanol vehicle for 48 h. Briefly, cells were homogenized in high salt KTED buffer (300 mM KCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM sodium molybdate, 10 mM benzamidine, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin, 1 mM PMSF) and ultacentrifuged 55,000 rpm for 45 min at 4 °C. The supernatants containing soluble nuclear proteins were desalted and concentrated using a Millipore Ultrafree centrifugal filter device (Fisher Scientific) with a nominal molecular weight limit of 10,000. The nuclear extract was incubated with 5 μl 10× phosphatase buffer and 30 units calf intestinal alkaline phosphatase (CIAP; Promega Corp., Madison, WI) in a 50 μl reaction volume and incubated for 1 h at 37 °C [12]. The mock reactions did not contain enzyme. Phosphatase reactions were stopped by addition of 4× Laemmli sample buffer and 5% β-mercaptoethanol, and subjected to electrophoresis as described above.

2.7. Electromobility shift assay

For binding reactions, 10 μg nuclear protein, prepared as described [11], and 50 μg/ml poly(dI-dC) were incubated in band shift buffer (10 mM Tris–HCl, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 2% glycerol). Reactions were initiated by the addition of oligonucleotide (p21 Vitamin D_3 response element (VDRE) radiolabeled to high specific activity), and incubated for 45 min. After addition of 4× stop buffer (50% glycerol and 0.1% bromophenol blue in 5× band shift buffer), the entire incubations were run on 4% polyacrylamide non-denaturing gels, dried, and exposed to X-ray film overnight.

2.7.1. Luciferase assay

Parental MCF-7 or MCF-7^{D_3Res} cells were plated in six-well dishes at a density of 2.5×10^5 cells per well in F-12 medium (Life Technologies) with 5% charcoal-stripped serum (CSS; Hyclone, Logan, UT) containing antibiotics. Cells were pretreated for 24 h with ethanol or 0.1–10 nM TPA. The cells were then washed, transfected (FuGENE6; Roche, Indianapolis, IN) with either pGL3B (empty vector) or pGL3B–24-hydroxylase (provided by Dr. Ohmdahl, University of New Mexico, Albuquerque, NM), and pRLSV40 (Promega, Madison, WI) for normalization of transfection efficiency. After an 18 h incubation with ethanol or 100 nM 1,25-$(OH)_2D_3$, the cells were lysed and analyzed using the dual-luciferase reporter assay system (Promega), and a Wallac Victor^2 1420 multilabel counter (Perkin-Elmer Life Sciences, Wellesley, MA).

2.8. Statistical evaluation

Data are expressed as mean ± S.E. One-way ANOVA was used to assess statistical significance between means. Differences between means were considered significant if *P*-values less than 0.05 were obtained with the Bonferroni method using GraphPad Instat software (GraphPad Software, San Diego, CA).

3. Results

3.1. Induction of apoptosis by 1,25-$(OH)_2D_3$ involves disruption of mitochondrial function

Intracellular signaling events downstream of the VDR were examined to determine the temporal changes in mitochondrial function after 1,25-$(OH)_2D_3$ treatment. We previously reported that redistribution of cytochrome *c* from mitochondria to cytosol is detected as early as 48 h after 1,25-$(OH)_2D_3$ treatment and precedes apoptotic morphology in MCF-7 cells [10]. Since exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen, we performed time course studies on mitochondrial ROS generation using flow cytometry. Production of superoxide anion was indirectly assessed as oxidation of hydroethidine to ethidium, which fluoresces red in cells due to its ability to bind DNA. As demonstrated in Fig. 1A, ROS production was enhanced within 72 h of

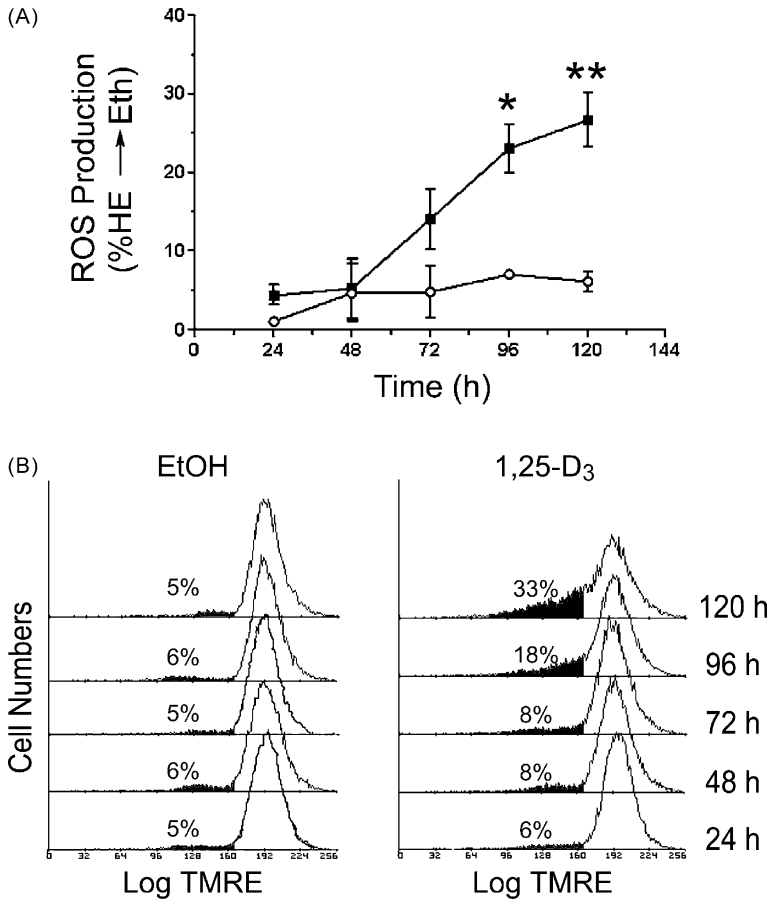

Fig. 1. Induction of apoptosis by 1,25-$(OH)_2D_3$ involves disruption of mitochondrial function. (A) ROS production. MCF-7 cells were treated with ethanol (○) or 100 nM 1,25-$(OH)_2D_3$ (■) and generation of ROS was assessed by flow cytometry at the indicated time points as described under Section 2. The data represent the percentage of cells positive for ROS and are expressed as mean ± S.E. of two independent experiments. (B) Mitochondrial membrane potential. MCF-7 cells were treated as described above and loss of mitochondrial membrane potential was analyzed by flow cytometry at the indicated time points as described under Section 2. Data are expressed as the percentage of cells with reduced mitochondrial membrane potential. The results are representative of at least three independent experiments. (*) $P < 0.05$; (**) $P < 0.01$; ethanol control vs. treated.

1,25-$(OH)_2D_3$ treatment in MCF-7 cells and continued to increase over time. To further probe mitochondrial function, the membrane potential-sensitive probe TMRE was used to detect mitochondrial membrane potential by flow cytometry. Loss of mitochondrial membrane potential was observed after 96 h, a late event during 1,25-$(OH)_2D_3$-mediated apoptosis (Fig. 1B).

Release of cytochrome *c* is considered to be a commitment point for activation of apoptosis since its release triggers caspase activation downstream of mitochondria through binding to Apaf-1 and autoactivation of procaspase-9. The mechanism of cytochrome *c* release into the cytosol across the mitochondrial outer membrane is not yet known, but the involvement of the Bcl-2 family of apoptotic regulators in this process is clear [8,9,13,14]. To study the role of the Bcl-2 family of proteins in 1,25-$(OH)_2D_3$-mediated apoptosis, fluorescence-activated cell sorting of annexin V-FITC was used to obtain populations enriched in viable or apoptotic cells. MCF-7 cells exhibiting apoptotic morphology such as pyknotic, irregularly shaped nuclei and cytosolic vacuolization also displayed externalization of phosphatidylserine as assessed by positive annexin V-FITC staining (Fig. 2A, see also Fig. 4). Loss of cytosolic Bax and enrichment of membrane bound Bax was observed in the apoptotic population isolated from 1,25-$(OH)_2D_3$-treated cells. Furthermore, the pro-apoptotic 18 kDa cleavage fragment of membrane bound Bax was exclusively present in the annexin V positive population of 1,25-$(OH)_2D_3$-treated cells, and not in the control or 1,25-$(OH)_2D_3$-treated viable populations [15]. Down-regulation of Bcl-2 was also observed in the 1,25-$(OH)_2D_3$-treated apoptotic cell population (Fig. 2B).

3.2. TPA can sensitize the Vitamin D_3-resistant variant to the effects of 1,25-$(OH)_2D_3$ on mitochondrial function and apoptosis

We have reported that the phorbol ester TPA, a PKC activator, can amplify the effects of 1,25-$(OH)_2D_3$ on induction of apoptosis in MCF-7 cells, and that TPA can partially

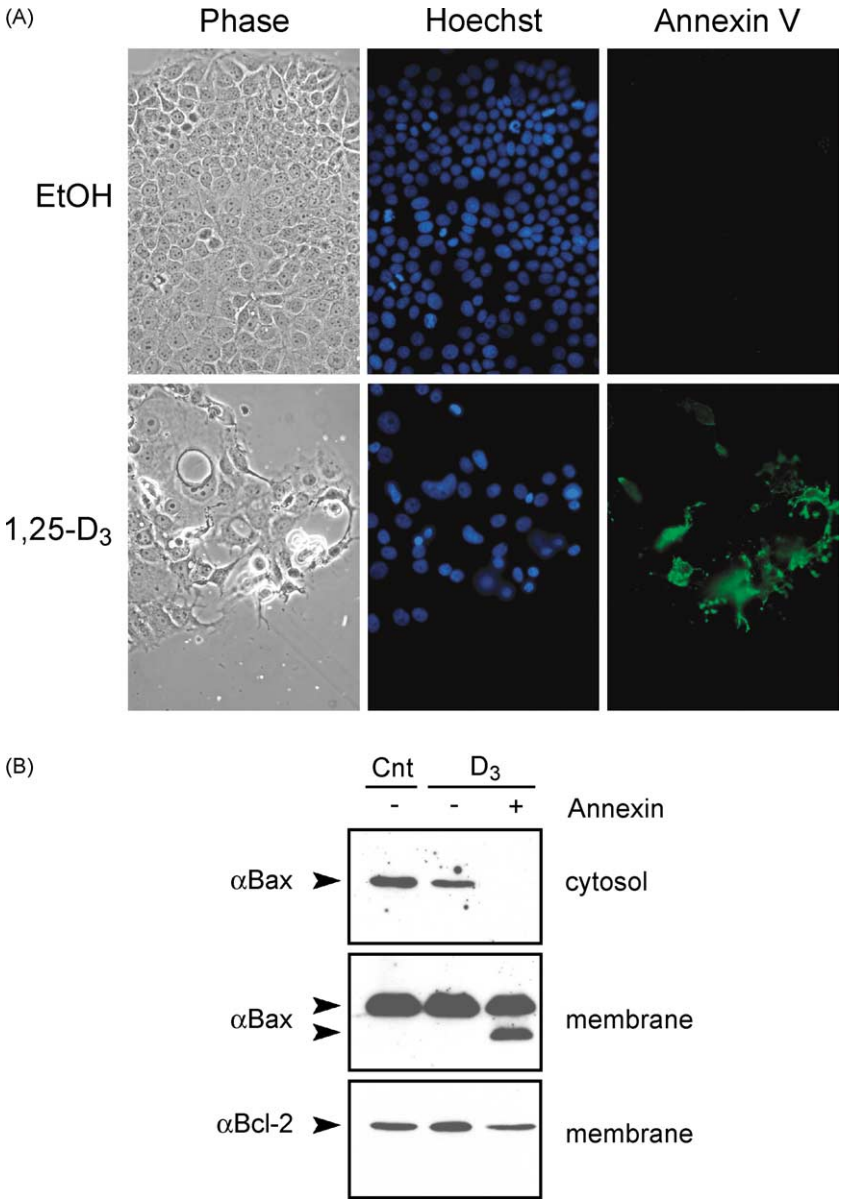

Fig. 2. Subcellular distribution of Bax and Bcl-2 after treatment with 1,25-$(OH)_2D_3$ in fluorescence-activated cell sorted populations. (A) Immunofluorescence. MCF-7 cells grown on Lab-Tek II chamber slides were treated with ethanol or 100 nM 1,25-$(OH)_2D_3$ for 5 days, incubated with annexin V-FITC and nuclei were counterstained with Hoechst 33258. The images were taken with an Olympus AX70 fluorescence microscope (magnification, 400×). *Left panels*, phase contrast; *middle panels*, Hoechst (*blue*); *right panels*, annexin V (*green*). (B) Subcellular distribution of Bax and Bcl-2. MCF-7 cells were treated with ethanol or 100 nM 1,25-$(OH)_2D_3$ for 5 days. Adherent and floating cells were harvested by trypsinization and pelleted, incubated with annexin V-FITC, and sorted into viable (annexin negative) and apoptotic (annexin positive) populations. The sorted populations were further fractionated into cytosolic and membrane fractions as described under Section 2, separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with Bax or Bcl-2 antibodies. The results are representative of at least two independent experiments.

sensitize the Vitamin D_3-resistant variant to the effects of 1,25-$(OH)_2D_3$ [4]. These data suggest an important role for phosphorylation pathways in dictating sensitivity to Vitamin D_3-mediated apoptosis. To determine whether TPA affected mitochondrial events involved in 1,25-$(OH)_2D_3$-mediated apoptosis of MCF-7 cells, mitochondrial membrane potential was assessed in cells treated with 100 nM 1,25-$(OH)_2D_3$ in the presence or absence of 1 nM TPA. As demonstrated in Fig. 3, MCF-7 cells treated with either 1,25-$(OH)_2D_3$ or TPA exhibited decreased mitochondrial membrane potential, and co-treatment with both compounds had a pronounced synergistic effect. These data suggest that phosphorylation pathways enhance the effect of 1,25-$(OH)_2D_3$ on mitochondrial-mediated apoptosis signaling.

In previous studies, we showed that neither cytochrome *c* release, Bax translocation or ROS production occur in 1,25-$(OH)_2D_3$-treated MCF-7^{D_3Res} cells [10]. Mitochondrial membrane potential was measured in MCF-7^{D_3Res}

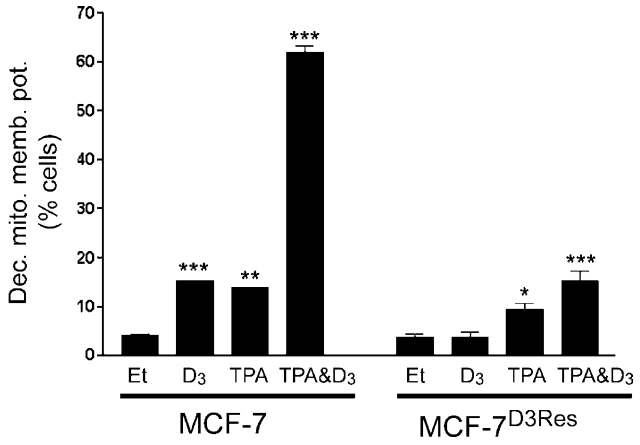

Fig. 3. TPA sensitizes the parental MCF-7 and MCF-7^{D_3Res} cells to the effects of 1,25-$(OH)_2D_3$ on mitochondrial membrane potential. Cells were treated with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA or both for 6 days. Loss of mitochondrial membrane potential was analyzed by flow cytometry as described under Section 2. Data represent the percentage of cells with reduced mitochondrial membrane potential, and are expressed as mean ± S.E of at least two independent experiments. (✱) $P < 0.05$; (✱✱) $P < 0.01$; (✱✱✱) $P < 0.001$; ethanol control vs. treated.

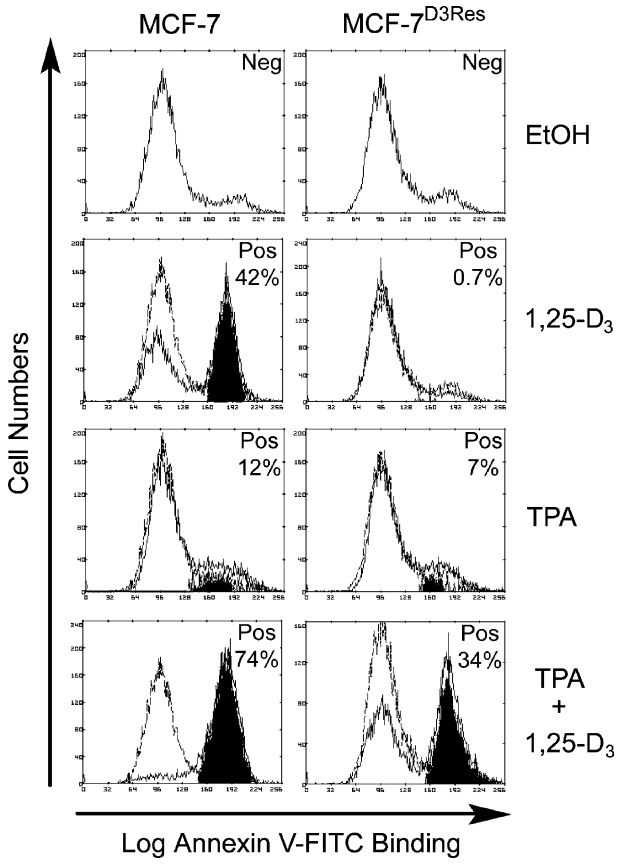

Fig. 4. TPA enhances 1,25-$(OH)_2D_3$-mediated apoptosis in parental MCF-7 and MCF-7^{D_3Res} cells. Cells were treated with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA or both for 6 days. Externalization of phosphatidylserine was assessed by flow cytometry as described under Section 2. Data are expressed as the percentage of annexin V-FITC positive cells after negative subtraction of data generated with vehicle-treated cells. The results are representative of a least two independent experiments.

cells treated with 100 nM 1,25-$(OH)_2D_3$ in the presence or absence of 1 nM TPA to determine whether TPA could sensitize MCF-7^{D_3Res} cells to the mitochondrial effects of 1,25-$(OH)_2D_3$. As shown in Fig. 3, when the MCF-7^{D_3Res} cells were treated with 1,25-$(OH)_2D_3$ in the presence of TPA, loss of mitochondrial membrane potential comparable to that induced by 1,25-$(OH)_2D_3$ in parental MCF-7 cells was observed.

External display of phosphatidylserine was used as an apoptotic marker to determine whether TPA enhanced 1,25-$(OH)_2D_3$-mediated effects on apoptosis in parental MCF-7 and MCF-7^{D_3Res} cells. As presented in Fig. 4, MCF-7 cells treated with 1,25-$(OH)_2D_3$ exhibited phosphatidylserine exposure, which was greatly enhanced in the presence of TPA. In contrast, 1,25-$(OH)_2D_3$ treatments had no effect on phosphatidylserine exposure in the Vitamin D_3-resistant variant, but TPA treatment sensitized the MCF-7^{D_3Res} cells to the effects of 1,25-$(OH)_2D_3$ by inducing external display of phosphatidylserine to levels similar to that induced by 1,25-$(OH)_2D_3$ in parental MCF-7 cells. This demonstrates that TPA can sensitize the Vitamin D_3-resistant variant to the effects of 1,25-$(OH)_2D_3$ on induction of apoptosis.

To further expand on these findings, subcellular localization of cytochrome *c* and Bax were examined by fluorescence microscopy in the parental MCF-7 and MCF-7^{D_3Res} cells treated with 1,25-$(OH)_2D_3$ in the presence or absence of TPA. In Fig. 5A and B, cytochrome *c* (*bottom panels*) and Bax (*middle panels*) staining is presented beside phase contrast images (*top panels*) to compare cytochrome *c* and Bax localization in individual viable and apoptotic cells. In vehicle-treated parental MCF-7 cells, apoptotic morphology was not present; Bax staining was diffuse and cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization. After treatment with 1,25-$(OH)_2D_3$ in the presence or absence of TPA in parental MCF-7 cultures, cells identified as apoptotic by phase contrast exhibited nuclear and cytoplasmic condensation, and cytosolic vacuolization. In these apoptotic cells, diffuse cytoplasmic cytochrome *c* staining was detected throughout the cell, which obscured the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm. In contrast, Bax exhibited punctate staining in these apoptotic cells, demonstrating the translocation of Bax from the cytosol to the mitochondria. Punctate Bax staining was also observed in cells which still retained mitochondrial cytochrome *c* indicating that translocation of Bax to mitochondria precedes cytochrome *c* release, and is one of the initiating events leading to disruption of mitochondrial activity. TPA-treated MCF-7 cultures contained many giant cells with large, round nuclei and secretory granules. Bax staining remained diffuse indicating cytosolic localization whereas cytochrome *c* staining was distributed mainly in the mitochondria, thus

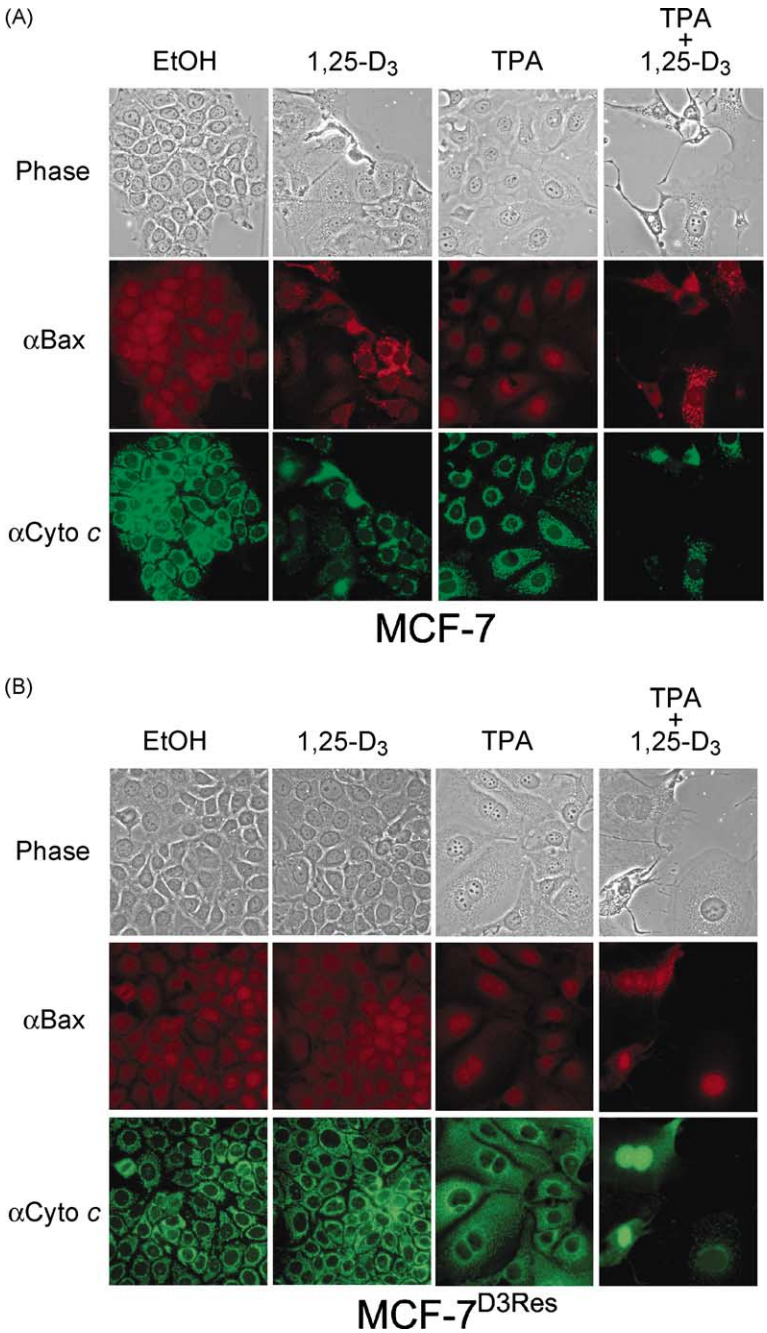

Fig. 5. Morphology, Bax translocation, and cytochrome *c* release in parental MCF-7 and MCF-7^{D_3Res} cells treated with 1,25-$(OH)_2D_3$ or TPA. Parental MCF-7 (A) and MCF-7^{D_3Res} (B) cells were grown on Lab-Tek II chamber slides, treated with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA or both, and fixed after 4 days. The fixed cells were immunostained with Bax and cytochrome *c* antibodies as described under Section 2, and nuclei were counterstained with Hoechst 33258. *Top panels*, phase contrast; *middle panels*, Bax (*red*); *bottom panels*, cytochrome *c* (*green*); magnification, 400×. The results are representative of at least two independent experiments.

demonstrating that TPA on its own did not induce redistribution of cytochrome *c* or Bax.

In MCF-7^{D_3Res} cells treated with vehicle or 1,25-$(OH)_2D_3$, apoptotic morphology was not present; Bax staining was diffuse and cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization. TPA-treated MCF-7^{D_3Res} cells displayed morphology which was similar to that of TPA-treated parental MCF-7 cells. However, when MCF-7^{D_3Res} cells were treated with 1,25-$(OH)_2D_3$ in the presence of TPA, the cells displayed apoptotic morphology and redistribution of both cytochrome *c* and Bax (Fig. 5B). This demonstrates that the effects of 1,25-$(OH)_2D_3$ on mitochondrial disruption and cell death may be modulated by activators of PKC.

3.3. Effect of 1,25-$(OH)_2D_3$ and TPA on VDR protein expression

To determine whether differences in response to 1,25-$(OH)_2D_3$ and TPA in parental MCF-7 and MCF-7^{D_3Res} cells are associated with differences in VDR protein expression, immunoblotting was performed on nuclear extracts isolated after treatment with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA or both for 48 h. As demonstrated in Fig. 6A, the VDR antibody (clone 9A7γ) recognized up to three closely spaced bands at approximately 50 kDa. The doublet or triplet bands may represent different phosphorylated forms [12] or the lower band may represent a subpopulation of the receptor that is targeted for degradation [16]. Consistent with our previous report [11], 1,25-$(OH)_2D_3$ up-regulated VDR protein expression in both parental MCF-7 and MCF-7^{D_3Res} cells, although appearance of an additional upper band was observed in the MCF-7 cells. Treatment of parental MCF-7 cells with TPA lead to up-regulation of the upper band and potentiated the effects of 1,25-$(OH)_2D_3$ by up-regulation of all three bands. In MCF-7^{D_3Res} cells, VDR was up-regulated by TPA and in the presence of both compounds, induction was enhanced compared to either compound alone. The MCF-7^{D_3Res} cells predominantly expressed the upper band of the VDR, thus indicating differences in VDR protein expression between the two cell lines.

A phosphatase experiment was performed to determine whether the multiple bands observed in VDR immunoblots reflect phosphorylation of the receptor. Nuclear extracts of parental MCF-7 and MCF-7^{D_3Res} cells treated with 1,25-$(OH)_2D_3$ or TPA were prepared in buffer without phosphatase inhibitors and incubated in the presence or absence of CIAP. Upon exposure to CIAP, the slower migrating forms of the receptor were either reduced or converted to the faster migrating form (Fig. 6B), suggesting that these bands represent different phosphorylated forms of VDR. This observation is consistent with previous reports that have demonstrated that the VDR is post-translationally modified by phosphorylation [12,17]. Furthermore, the extent of VDR phosphorylation is altered in the presence of 1,25-$(OH)_2D_3$ or TPA (Fig. 6A and B).

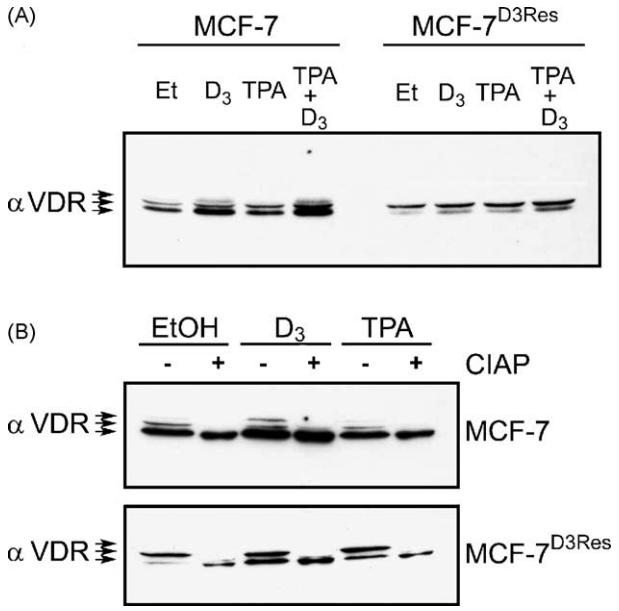

Fig. 6. VDR protein expression in parental MCF-7 and MCF-7^{D_3Res} cells treated with 1,25-$(OH)_2D_3$ or TPA. (A) VDR protein expression. Nuclear extracts derived from cells treated for 48 h with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA or both were solubilized in Laemmli buffer, separated on SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with VDR antibody. The results are representative of at least three independent experiments. (B) Phosphatase treatment. Nuclear extracts derived from cells treated as described above but in the absence of phosphatase inhibitors were incubated in the presence or absence of CIAP for 1 h at 37 °C. Reactions were stopped by solubilization in Laemmli buffer and processed as described above. The results are representative of at least three independent experiments.

3.4. Effect of TPA on VDR binding to DNA and transactivation ability

To determine whether TPA altered VDR binding to the VDRE on DNA, nuclear extracts of MCF-7 cells treated with 1,25-$(OH)_2D_3$ or TPA were prepared in the presence of protease and phosphatase inhibitors and incubated with a radiolabeled oligonucleotide corresponding to the VDRE from the human p21 gene [18]. Two major DNA–protein complexes were generated when extracts from MCF-7 cells were incubated with a radiolabeled

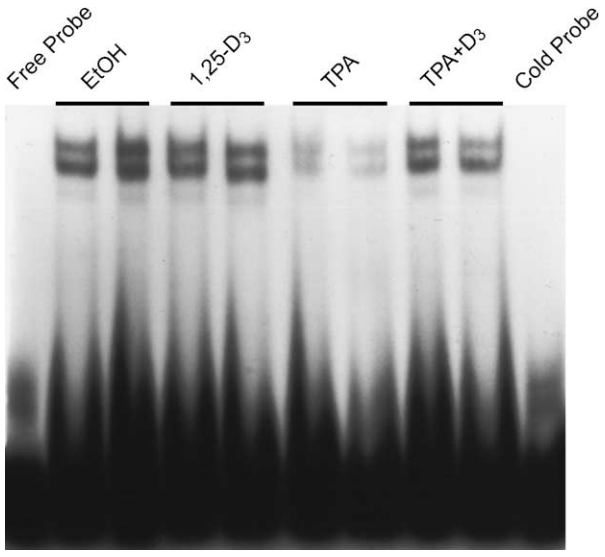

Fig. 7. Electromobility shift analysis of VDR binding to human p21 VDRE using nuclear extracts of parental MCF-7 cells treated with 1,25-$(OH)_2D_3$ or TPA. Nuclear extracts were isolated from parental MCF-7 cells treated with ethanol, 100 nM 1,25-$(OH)_2D_3$, 1 nM TPA, or both for 48 h. The nuclear extracts were incubated in duplicate with ^{32}P-labeled oligonucleotide corresponding to human p21 VDRE as described under Section 2. DNA–protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. The results are representative of at least two independent experiments.

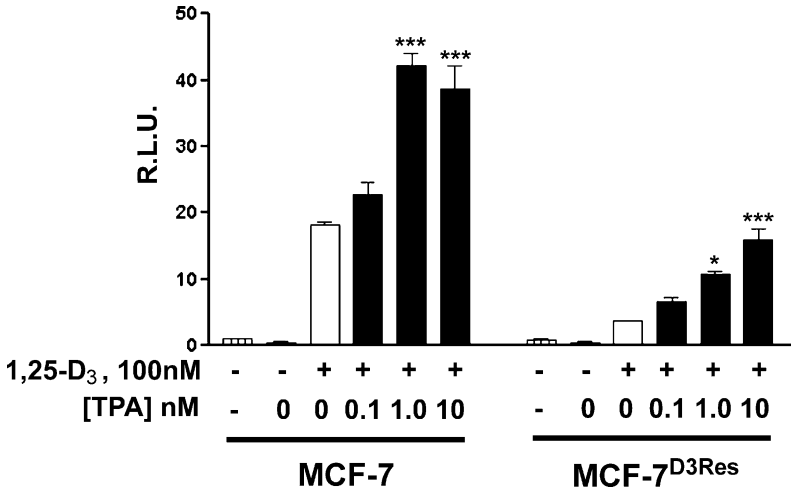

Fig. 8. TPA potentiates 1,25-$(OH)_2D_3$-induced transcriptional activity of the VDR in parental MCF-7 and MCF-7^{D_3Res} cells. Cells grown in phenol-red free media containing charcoal-stripped serum were pretreated with ethanol or 0.1–10 nM TPA for 24 h. Cells were then transfected with either pGL3B (empty vector) or pGL3B–24-hydroxylase luciferase constructs and treated with ethanol or 100 nM 1,25-$(OH)_2D_3$ for 18 h. Relative luciferase units (RLU) have been corrected for transfection efficiency with cotransfected pGL3–SV40 using the dual-luciferase assay and expressed relative to values obtained with pGL3B empty vector. Data are expressed as mean ± S.E. of three wells, and are representative of at least two independent experiments. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; ethanol control vs. treated.

oligonucleotide containing the p21 VDRE (Fig. 7). Treatment of cells with 1,25-$(OH)_2D_3$ did not alter the pattern or intensity of DNA protein complexes. In MCF-7 cells treated with TPA, however, DNA–protein complexes were barely detectable, consistent with TPA-mediated phosphorylation pathways inhibiting VDR binding to DNA [7,17]. When cells were treated with TPA and 1,25-$(OH)_2D_3$, DNA binding activity was restored to levels similar to that observed in control or 1,25-$(OH)_2D_3$-treated cells. Thus, VDR binding to sequence-specific DNA is not abrogated by TPA in the presence of 1,25-$(OH)_2D_3$. These data demonstrate that TPA modulated phosphorylation pathways affect DNA binding of the VDR (Fig. 7).

We next assessed transcriptional activity of the VDR after treatment with TPA in the presence or absence of 1,25-$(OH)_2D_3$ by reporter gene assay with a 24-hydroxylase promoter luciferase construct. This promoter contains two VDREs and is highly inducible by 1,25-$(OH)_2D_3$ in cells that contain functional VDR. Basal transcriptional activity of the 24-hydroxylase was similar in parental MCF-7 and MCF-7^{D_3Res} cell lines, however, 1,25-$(OH)_2D_3$ stimulated 24-hydroxylase luciferase activity was blunted in MCF-7^{D_3Res} cells compared to parental MCF-7 cells (Fig. 8). TPA pretreatment (1–10 nM) enhanced 1,25-$(OH)_2D_3$ stimulated 24-hydroxylase luciferase activity in parental MCF-7 cells (up to two-fold induction). In MCF-7^{D_3Res} cells, TPA pretreatment dose dependently enhanced 1,25-$(OH)_2D_3$ stimulated 24-hydroxylase luciferase activity, with a four-fold increase achieved with 10 nM TPA. Induction of 24-hydroxylase reporter gene activity in MCF-7^{D_3Res} cells treated with 1,25-$(OH)_2D_3$ in the presence of 10 nM TPA was similar to that observed in 1,25-$(OH)_2D_3$-treated parental MCF-7 cells. However, maximal induction remained lower in MCF-7^{D_3Res} than the parental MCF-7 cells in the presence of both 1,25-$(OH)_2D_3$ and TPA. Although further studies are necessary to identify the underlying basis for reduced transcriptional activity of the VDR in MCF-7^{D_3Res} cells, our data implicates TPA modulated phosphorylation pathways in dictating cell sensitivity to 1,25-$(OH)_2D_3$ at the level of the VDR.

4. Discussion

In these studies, we have explored the role of mitochondrial disruption in 1,25-$(OH)_2D_3$-mediated apoptosis and demonstrate that TPA targeted phosphorylation pathways can modulate breast cancer cell sensitivity to 1,25-$(OH)_2D_3$-mediated apoptosis. Here we provide direct evidence for involvement of Bcl-2 apoptotic regulators in mitochondrial disruption during 1,25-$(OH)_2D_3$-mediated apoptosis of MCF-7 cells. Immunoblot analysis demonstrates loss of cytosolic Bax, enrichment of membrane bound Bax, and down-regulation of Bcl-2 in the apoptotic MCF-7 cell population generated by treatment with 1,25-$(OH)_2D_3$. In addition, immunocytofluorescence evidence indicates redistribution of Bax from cytosol to mitochondria after treatment with 1,25-$(OH)_2D_3$ in MCF-7 cells, but not in Vitamin D_3-resistant cells. Furthermore, in MCF-7 cells, translocation of Bax to mitochondria always precedes cytochrome *c* release and thus appears to be an initiating event that leads to disruption of mitochondrial activity. These data complement our previous studies and lend additional support to the concept that 1,25-$(OH)_2D_3$-mediated apoptosis is driven by Bax

translocation [10]. This view is consistent with earlier reports that overexpression of Bcl-2 blocks 1,25-$(OH)_2D_3$-induced apoptosis in both breast and prostate cancer cells [19,20]. Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data collectively imply that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1,25-$(OH)_2D_3$-mediated apoptosis. The nature of the signals generated by 1,25-$(OH)_2D_3$ that induce Bax translocation to mitochondria are currently unknown. Since events upstream of Bax translocation to mitochondria in response to 1,25-$(OH)_2D_3$ are abrogated in the Vitamin D_3-resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

Time course studies demonstrate that cytochrome *c* release and production of ROS are relatively early events in 1,25-$(OH)_2D_3$-mediated apoptosis of MCF-7 cells since these events occur before any widespread morphological markers of apoptosis are observed. Several studies have shown that cytochrome *c* release can proceed in the absence of mitochondrial transmembrane potential dissipation, suggesting that some cytochrome *c* remains in the mitochondria or that it may freely re-enter the intermembrane space and participate in electron transport [21]. In some experimental systems (i.e. UV or staurosporine treatment of CEM cells), caspase inhibitors are sufficient to maintain mitochondrial membrane potential after the release of cytochrome *c* indicating that the loss of mitochondrial membrane potential is caspase-dependent [22]. However, we have shown in a previous study that 1,25-$(OH)_2D_3$-mediated loss of mitochondrial membrane potential and ROS production is caspase-independent [10]. ROS production induced by 1,25-$(OH)_2D_3$ may potentiate the decrease in mitochondrial membrane potential by removing electrons from the electron transport chain in a caspase-independent manner [23]. Hence, in our experimental system, loss of mitochondrial membrane potential appears to be a late event triggered by ROS production.

TPA is a potent inhibitor of breast cancer cell growth that induces growth arrest in G_2/M, up-regulation of p21, and accumulation of the hypophosphorylated form of retinoblastoma protein [4,24]. However, we have previously shown that these effects of TPA are not sufficient to mediate apoptosis in MCF-7 cells. In the present studies, we confirm that neither Bax translocation or cytochrome *c* release is induced in MCF-7 cells treated with TPA alone. Thus, the reduction in mitochondrial membrane potential detected after TPA treatment in both parental MCF-7 and MCF-7^{D_3Res} cells is apparently not of sufficient magnitude or duration to permit redistribution of cytochrome *c* and apoptosis. This observation is consistent with data obtained in isolated mitochondria treated with an uncoupler (carbamoyl cyanide *n*-chlorophenylhydrazone), which induces a rapid decrease in mitochondrial membrane potential but fails to elicit cytochrome *c* release or apoptosis [25]. However, the reduced mitochondrial membrane potential induced by TPA may sensitize cells to mitochondrial apoptosis, as treatment of MCF-7 cells with both TPA and 1,25-$(OH)_2D_3$ induces apoptosis to a much greater extent than 1,25-$(OH)_2D_3$ alone. Cultures treated with TPA and 1,25-$(OH)_2D_3$ exhibit phosphatidylserine exposure, decreased mitochondrial membrane potential, translocation of Bax and cytochrome *c* release at a higher frequency than 1,25-$(OH)_2D_3$-treated cultures. Moreover, TPA potentiates the effects of 1,25-$(OH)_2D_3$ in the Vitamin D_3-resistant MCF-7 variant, resulting in reversal of 1,25-$(OH)_2D_3$ resistance, and apoptosis induction at a level similar to that observed in 1,25-$(OH)_2D_3$-treated MCF-7 cells.

Regulation of VDR by TPA, an activator of PKC, suggests cooperativity between the PKC signal transduction pathway and 1,25-$(OH)_2D_3$ action. Mechanisms may involve effects on VDR abundance, VDR binding to its target genes, and/or on modulation of general transcription factors or coactivators that interact with VDR to mediate 1,25-$(OH)_2D_3$-induced transcriptional activity [17,26]. Exposure of parental MCF-7 and MCF-7^{D_3Res} cells to TPA augments their response to 1,25-$(OH)_2D_3$, and this may be due to changes in the extent of receptor phosphorylation and up-regulation of VDR protein levels. Studies have demonstrated that TPA can potentiate the effects of 1,25-$(OH)_2D_3$ on gene expression [27]. For example, Armbrecht et al. [28] demonstrated that TPA, in the presence of 1,25-$(OH)_2D_3$, markedly up-regulated 24-hydroxylase mRNA expression in intestinal IEC cells, and the ability of PKC inhibitors to block the activity of both compounds suggests that 1,25-$(OH)_2D_3$ and TPA act through PKC-dependent pathways. In this study, we show that TPA pretreatment greatly enhances 1,25-$(OH)_2D_3$ stimulation of the 24-hydroxylase promoter. Moreover, in the MCF-7^{D_3Res} cells, TPA markedly increases the ability of 1,25-$(OH)_2D_3$ to stimulate 24-hydroxylase promoter activity, restoring VDR transactivation to levels comparable with those detected in 1,25-$(OH)_2D_3$-treated parental MCF-7 cells. Hence, interactions with signal transduction pathways can enhance 1,25-$(OH)_2D_3$ induction of target gene expression and changes in cellular phosphorylation may play a significant role in determining the effectiveness of 1,25-$(OH)_2D_3$ on transcriptional control [29]. Our data support the concept that regulation of VDR by signal transduction pathways may play an important role in modulating target cell responsiveness to 1,25-$(OH)_2D_3$-mediated growth inhibition and apoptosis.

In summary, 1,25-$(OH)_2D_3$-mediates apoptosis of MCF-7 cells through mitochondrial signaling, which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Most significantly, the observation that TPA can partially sensitize the Vitamin D_3-resistant variant to the effects of 1,25-$(OH)_2D_3$ suggests an important role for phosphorylation in dictating sensitivity to Vitamin D_3-mediated apoptosis. These data indicate that the effects of 1,25-$(OH)_2D_3$ on mitochondrial disruption and apoptosis might be sensitized through activators of PKC.

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