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Antiestrogenic Effects of All-*trans*-Retinoic Acid and 1,25-Dihydroxyvitamin D_3 in Breast Cancer Cells Occur at the Estrogen Response Element Level but through Different Molecular Mechanisms

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ABSTRACT

Most breast tumors show estrogen-dependent growth and are thus susceptible to antiestrogenic therapy. MCF-7 cells, obtained from a human estrogen-dependent breast carcinoma, are widely used for studying the modulation of estrogenic responses by different effectors. All-*trans*-retinoic acid (RA) and 1,25-dihydroxyvitamin D_3 (Vit D_3) inhibited estrogen-induced growth of MCF-7 cells and their effect was potentiated by the classical antiestrogen, hydroxytamoxifen. In MCF-7 cells, we found that RA and Vit D_3 also inhibited estrogen-induced transcription; this was shown both for an endogenous gene (*pS2*) and for various exogenous transfected genes. Their inhibitory effect could not be reversed by increasing estradiol concentrations, showing that contrary to classical antiestrogens, they did not compete with estradiol to bind the estrogen receptor (ER).

Analysis of the inhibitory mechanisms indicates that RA and Vit D_3 receptors can directly or indirectly impair the binding of ER to the estrogen responsive element. The antagonist effect of RA would be found especially at DNA level since it seems to essentially involve an estrogen responsive element. The antagonist effect of Vit D_3 would be found especially at the ER level since it seems to concern estrogen binding and dimerization domains of ER.

We conclude that the antiestrogenic effects of RA and Vit D_3 are similar since they can, via their receptors, interfere with estrogenic action at the estrogen responsive element level but that they are not identical since different molecular mechanisms are involved.

INTRODUCTION

Nonsteroidal, antiestrogenic drugs are the normal choice of endocrine treatment for hormone-dependent breast carcinoma since they markedly improve survival rates and have few side effects (1, 2). Unfortunately, not all patients benefit from endocrine therapy. Moreover, as hormone deprivation produces cytostatic rather than cytotoxic effects, tumors evolve under selective pressure and can become resistant to endocrine therapy (3). Interchanging or combinatory use of other types of drugs with antiestrogenic effects might thus be an alternative to classical endocrine therapy.

RA[2] and Vit D_3 are known to induce differentiation and consequently inhibit the proliferation of several cell types (4–7). Their cellular activity is mediated by nuclear receptors which belong to a superfamily that includes steroid and thyroid hormone receptors (8, 9). Nuclear receptors regulate gene transcription in a ligand-dependent manner by recognizing specific DNA sequences called hormone-responsive elements, and this interaction modulates transcription of cognate-target genes involved in a wide variety of developmental and physiological phenomena (8, 9). Both response elements for RA and Vit D_3 have a direct repeat configuration of the 5′-(G/A)GGT(G/C)-A-3′ motif which is also shared by the palindromic estrogen and thyroid hormone response elements. Based on their similar recognition sites, these receptors have been classified in the same subfamily within the superfamily of nuclear receptors (10, 11). Inside this subfamily, RXR (12–18) has been shown to form heterodimers with several members of this subfamily. Such interactions were initially described with RAR, TR, and VDR and more recently with peroxisome proliferator receptors and chicken ovalbumin upstream promoter transcription factor (19–24). Such heterodimerization results in an increased transcriptional activity of the partner receptor and designates RXR as its cellular coregulator. In addition to heterodimer formation of RXR with RAR, TR, or VDR, other kinds of interactions at receptor or response element level have also been noted in this subfamily (25–27). These interactions result in positive or negative regulation of transcription, often in a cell- and promoter-specific manner. Taken together, these data indicate a complex pattern of regulation in which nuclear receptors are involved.

The present study aimed to investigate the interference of RA and Vit D_3 with estrogenic response pathways in hormone-dependent breast cancer cells. Here, in addition to classical responses (induction of cell growth and pS2 mRNA), we exploited MVLN cells (28) and analyzed some aspects of the inhibitory effects of RA and Vit D_3. These cells correspond to MCF-7 cells stably transfected with the reporter plasmid Vit-tk-Luc in which the 5′ flanking region of the *Xenopus* vitellogenin A2 gene that contains an ERE is inserted in front of the herpes simplex virus promoter for tk (29). This regulatory part controls the firefly Luc structural gene. We reported previously that in MVLN cells, the estrogenic response of the chimeric gene behaved like a natural estrogenic one, similar to the cellular growth or induction of the progesterone receptor in MCF-7 cells (30).

In this paper, we clearly demonstrate the inhibitory effect of RA and Vit D_3 on estrogen-regulated gene transcription and present evidence that RA and Vit D_3 receptors interact with estrogenic action at the ERE level but through different molecular mechanisms.

MATERIALS AND METHODS

Materials. Materials for cell culture and random priming kit came from Gibco-BRL (Cergy Pontoise, France). TPA, RA, luciferin, E2, dexamethasone, 4,6-diamidino-2-phenylindole, and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). OH Tam was from ICI (Macclesfield, United Kingdom). Vit D_3 was kindly provided by Dr. D. Philibert (Roussel Uclaf, Romainville, France). Effector solutions were prepared in ethanol except TPA, which was prepared in dimethyl sulfoxide. RNazol B reagent was from Bioprobe (Montreuil, France). [^{3}H]Estradiol (2.59 TBq/mmole), nylon transfer membrane Hybond N$^+$, and Hyperfilm MP were from Amersham (Les Ulis, France).

Cell Lines and Cell Culture Conditions. All cell lines (MVLN, MCF-7, and COS) were cultured in Dulbecco's modified Eagle's medium without phenol red and supplemented with 5% FCS. MVLN cells were described elsewhere (28, 30). Experiments were performed using medium supplemented

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[2] The abbreviations used are: RA, all-*trans*-retinoic acid; Vit D_3, vitamin D_3; RARE, retinoic acid response element; RXR, 9-*cis*-retinoic acid receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor; VDR, vitamin D_3 receptor; ERE, estrogen responsive element; tk, thymidine kinase; Luc, luciferase; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; E2, estradiol; OH Tam, 4-hydroxytamoxifen; FCS, fetal calf serum; ER, estrogen receptor; GR, glucocorticoid receptor; cass, cassette; ABCD, avidin-biotin complex DNA; ODN, oligodeoxynucleotide; WCE, whole-cell extract.

with 2% dextran-coated charcoal-treated FCS instead of FCS as in routine culture (31). Experiments with RA and Vit D_3 were performed under subdued light.

Cell Growth Assay. Effects of various concentrations of RA and Vit D_3 alone or in the presence of OH Tam (300 nM) were studied on the estrogen-dependent growth of MCF-7 cells as described previously (32). Cellular DNA content was determined by using fluorescent dye 4,6-diamidino-2-phenylindole method with calf thymus DNA as standard (33).

Plasmids and Transient Transfection. Vit-tk-Luc, ERE-βG-Luc, and $(17M)_5$-βG-Luc plasmids have been described previously (31). The $(TRE)_3$-tk-Luc plasmid was constructed by placing three repeated sequences of the AP-1 binding site (34) upstream from the tk promoter and luciferase structural gene. The plasmid Gal-ER encoded a chimeric Gal4-estrogen receptor in which the DNA binding domain of the yeast GAL4 protein (Gal) replaced the natural DNA binding domain of the steroid receptor and was fused to the natural DEF domains of the ER; this chimeric receptor stimulates luciferase transcription from the cotransfected reporter gene $(17M)_5$-βG-Luc. The HEG0, ER-GRcass, and GR-ERcass plasmids have been described previously (35) and encode ER, ER containing the C domain of the glucocorticoid receptor (GR), and GR containing the C domain of ER, respectively. The TAT-tk-Luc plasmid was constructed from the TAT-tk-CAT plasmid (36). Plasmids encoding the VDR, the RA receptor α, and the 9-*cis*-RA receptor α have been presented elsewhere (12, 16, 37). Standard protocols of recombinant DNA technology were used for plasmid preparations (38). Transfection experiments were performed according to the calcium phosphate coprecipitation procedure (38). At the end of incubation with various compounds, the luciferase activity was determined as described previously (31). Results were expressed as arbitrary units per mg of protein assayed by the method of Lowry *et al.* (39).

Northern Blot Experiments. MCF-7 cells were seeded to 2.5×10^6 cells per 100-mm dishes with a medium supplemented with 2% dextran-coated charcoal-treated FCS. One day later, cells received estradiol (10 nM) while control cells received the vehicle. Cells were incubated for 48 h with estradiol, and RA (1 μM) or Vit D_3 (100 nM) was added at various times (3, 6, 12, and 24 h). At the end of incubation, total RNA was isolated according to the method of Chomczynski and Sacchi (40) by using the RNazol B reagent. Total RNA (10 μg) was separated electrophoretically on a 1% agarose denaturing gel and transferred to a nylon membrane. The membrane was hybridized overnight with [^{32}]P-labeled pS2 and 36B4 probes (41) at 42°C in 50% formamide. After stringency washes, filters were exposed to a film with intensifying screens. Autoradiograms were scanned densitometrically to determine the relative amount of the pS2 mRNA to that of 36B4. Results are expressed as percentages with the pS2 mRNA level induced by 10 nM E2 taken as 100%.

Avidin-Biotin Complex DNA Binding (ABCD). Synthetic oligonucleotides, two 25-mer ODNs including the palindromic ERE (5′ gatccAGGTCAc(t or a)gTGACCTggatc 3′) of the vitellogenin A2 gene, were synthesized by using a DNA synthesizer (Applied Biosystems). ODNs were coupled at the 5′-phosphate end to an aminolinker II (Applied Biosystems). Preparation, purification, and annealing of biotinylated ODNs were carried out as described (42, 43).

In the ABCD assay, COS cells seeded to 2.5×10^6 cells per 100-mm dishes were transiently transfected with plasmids encoding VDR, RA receptor α, or ER. Seventy-two h after transfection, cells were incubated for 4 h with the corresponding ligand of the studied receptor [radioactive estradiol (3 nM), cold RA (1 μM), or cold Vit D_3 (100 nM)]. After incubation, cells were harvested and washed, and the various WCEs were prepared by potter homogenization at 4°C in a high salt buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 0.5 M KCl, 1 mM dithiothreitol, 5 μg/ml aprotinin, and 40 μg/ml phenyl methylsulfonyl fluoride. The homogenate was further incubated on ice for 1 h and then centrifuged at $10,000 \times g$ for 15 min to yield the WCE (supernatant). WCEs were then diluted four times with buffer A [50 mM KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol]. The various WCEs tested in an ABCD experiment were prepared at the same time giving a similar protein concentration and were immediately tested for their ability to bind a biotinylated ERE. The WCE containing ER labeled with tritiated estradiol (30 μl-200 fmole) was incubated with 30 μl WCE of untransfected COS cells (control) or with WCE of transfected COS cells containing VDR or RAR. The final molarity of each test tube was adjusted to 0.1 M KCl (final volume, 0.4 ml); various amounts of biotinylated ERE were then added, and the incubation was continued overnight at 4°C. Protein-DNA complexes were precipitated by addition of streptavidin-Sepharose. After a 2 h incubation period at 4°C, the pellet was washed three times with buffer A before determining its radioactivity content. Results are expressed in fmole ER bound to the biotinylated ERE (nM).

RESULTS

Effect of RA and Vit D_3 on Estrogen-induced MCF-7 Cell Growth

The growth of MCF-7 cells is one of the reference responses studied which characterizes the (anti)estrogenic effect of a compound. With this classical and complex estrogenic response, Vit D_3 and RA behaved as antagonist compounds since they were able to inhibit cell growth induced by 10 nM estradiol (Fig. 1). The inhibitory effects of RA and Vit D_3 occurred in a concentration-dependent manner with concentrations ranging from 3 to 300 nM for RA and from 0.3 to 30 nM for Vit D_3. By combining very low concentrations of RA or Vit D_3 (*e.g.*, 10 nM or 1 nM, respectively) with OH Tam (300 nM), a full inhibitory effect was obtained [a level attained with OH Tam (300 nM) in the absence of estradiol]. Under the same experimental conditions when tested alone in the presence of estradiol, each of these compounds lead only to a partial antiproliferative effect.

Effect of RA and Vit D_3 on Estrogen-induced Gene Expression

Effect of RA and Vit D_3 on Estrogen-induced pS2 mRNA. Because cell growth is a response involving several regulatory pathways and thus many genes, we investigated the inhibitory effect of Vit D_3 and RA on a single endogenous gene transcriptionally controlled by the ER. As shown in Fig. 2, Northern blot analysis of the pS2 mRNA level in MCF-7 cells showed that RA (1 μM), like Vit D_3 (100 nM), inhibited the estradiol-induced pS2 mRNA. The time-course of the RA effect revealed a rapid decrease in the pS2 mRNA level detectable

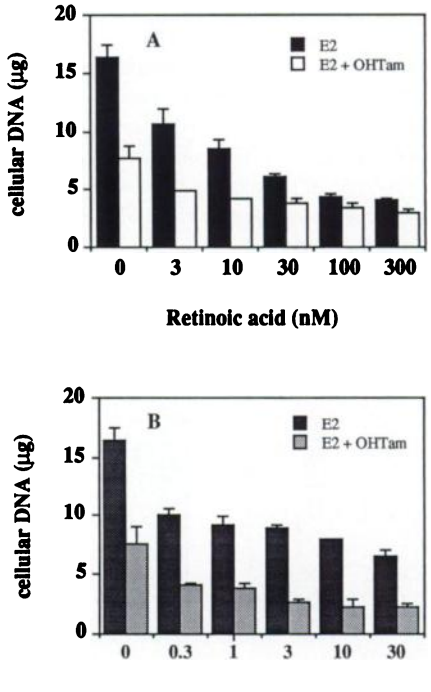

Fig. 1. Effect of increasing concentrations of RA and Vit D_3 on the estrogen-dependent growth of MCF-7 cells treated with 10 nM E2 in the presence or not of 300 nM OH Tam. The experiment was stopped when cells treated with E2 reached confluence. The DNA assay was then performed as described in "Materials and Methods." Experiments were performed in triplicate, and the results are expressed as a mean ± SE of μg DNA per well. The value obtained with OH Tam (300 nM) in the absence of estradiol is 3.5 ± 0.5 μg DNA.

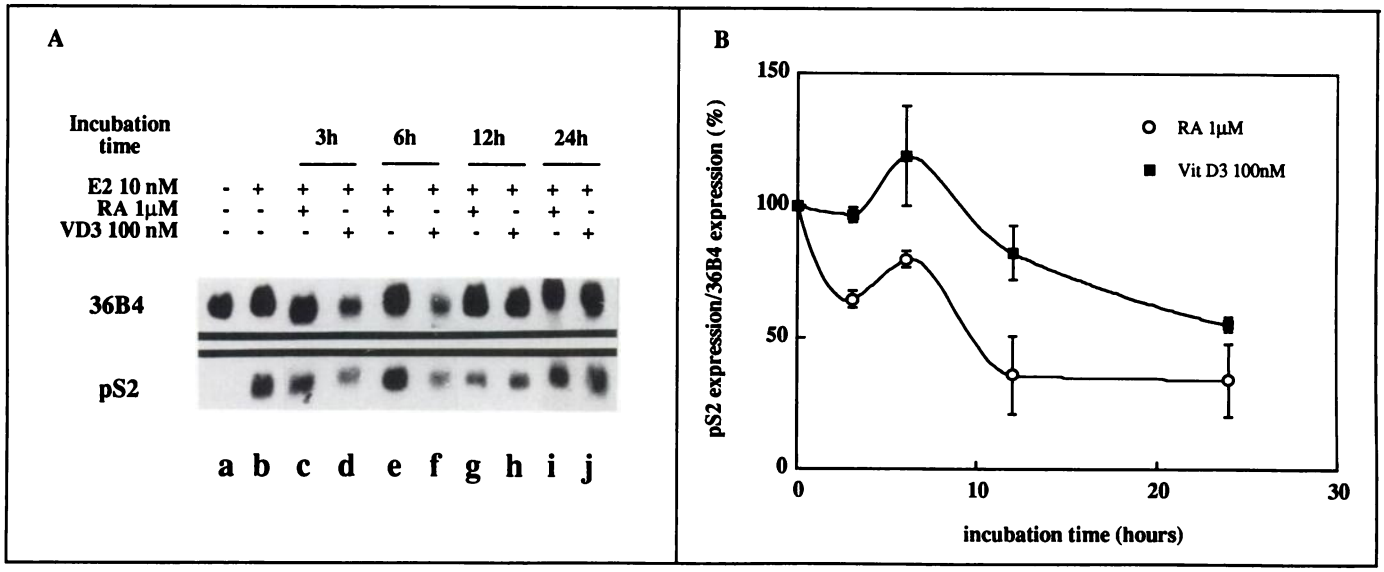

Fig. 2. Effect of RA and Vit D_3 on the estrogen-dependent induction of pS2 mRNA in MCF-7 cells. All cells were incubated for 48 h with 10 nM E2 except control cells, which received the vehicle (ethanol) alone. RA (1 μM) or Vit D_3 (100 nM) were added at various times. A Northern blot experiment was performed as described in "Materials and Methods." *(A)* autoradiogram of a representative experiment showing the RNA species corresponding to pS2 (0.6 kilobases) and 36B4 (1.2 kilobases). *(B)* autoradiograms from different experiments were densitometrically scanned to determine the relative amounts of pS2 mRNA. The results are expressed as a percentage ± SE taking the pS2 mRNA level induced by 10 nM E2 as 100%.

3 h after treatment of MCF-7 cells, and the effect was maximal after 12 h. The effect of Vit D_3 on the pS2 mRNA level was apparent 12 h after with a maximal effect after 24 h.

Study of a Chimeric Estrogen-induced Gene in a Stable Cell Line. The pS2 promoter involves a complex regulatory part (44) inducible by estrogens, growth factors, and some oncogene products. We thus investigated the effect of RA and Vit D_3 on a gene selectively regulated by ER by using MVLN cells, a cellular model corresponding to MCF-7 cells stably transfected with the Vit-tk-Luc plasmid. In this model in which the estrogenic response of the chimeric gene behaved like natural estrogenic ones (30), RA (Fig. 3*A*) and Vit D_3 (Fig. 3*B*) inhibited estradiol-induced luciferase expression. These inhibitory effects occurred in a concentration-dependent manner for concentrations ranging from 0.1 to 1 μM and 1 to 100 nM for RA and Vit D_3, respectively. Interestingly, inhibitory effects of RA and Vit D_3 could not be reversed by increasing the estradiol concentration.

As a control, the antagonistic effect of RA and Vit D_3 on the expressed estrogenic response did not involve any significant modulation of estradiol binding parameters. Scatchard analysis, performed under the same experimental conditions as those leading to the antagonistic effect of RA and Vit D_3, showed no significant modification in the estradiol dissociation constant for the ER or in the ER content (Ref. 31; data not shown). As observed above about cell growth, the inhibitory effect of RA and Vit D_3 on estrogen-dependent luciferase expression in MVLN cells was additive to that of OH Tam (data not shown).

Does the Antagonistic Effect of RA and Vit D_3 Occur at the ERE Level?

All of the above described results led us to study the mechanism of action of RA and Vit D_3 at the ERE level. This study was carried out using both ABCD and transient transfection experiments. ABCD experiments were performed using COS cells to express RAR, VDR, and ER. As shown in Fig. 4, under our experimental conditions, the different WCEs containing the expressed receptors (RAR or VDR)

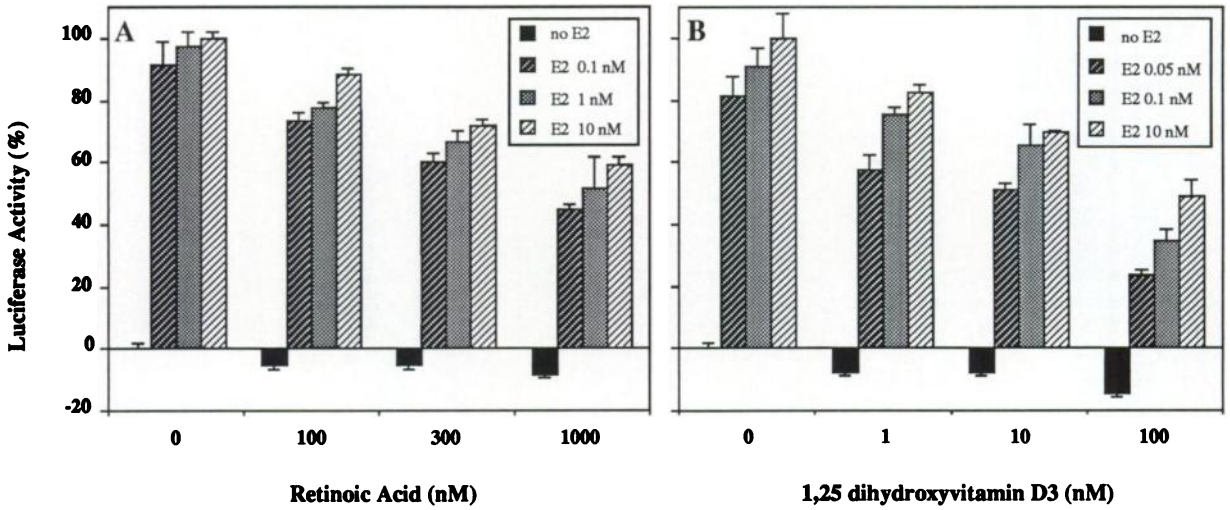

Fig. 3. Effect of RA and Vit D_3 on estrogen-dependent luciferase induction of MVLN cells. Cells were incubated with the vehicle (ethanol) or with different E2 concentrations in combination or not with various concentrations of RA *(A)* or Vit D_3 *(B)*. At the end of incubation (24 h) with effector(s), cells were treated as described in "Materials and Methods" to determine the luciferase activity per mg protein. The results are expressed as a mean percentage ± SE taking the luciferase activity induced by 10 nM estradiol as 100%.

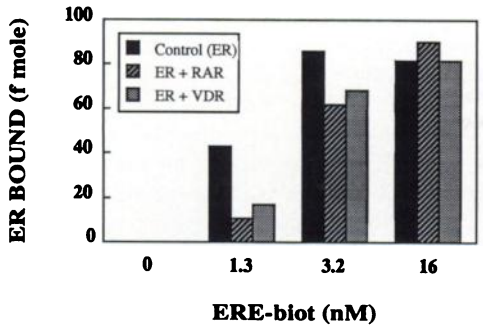

Fig. 4. As described in "Materials and Methods," the ABCD experiment was realized by expressing ER, VDR, and RAR in COS cells and by preparing WCEs from cells previously incubated with the corresponding ligand. The ER (WCE), radiolabeled using radioactive E2 (200 fmole), was incubated with WCE of untransfected COS cells (control) or with WCE of transfected COS cells (VDR or RAR). As described, the different WCEs were immediately tested for their ability to bind a biotinylated ERE. The results are expressed in fmole ER bound to the ERE. The figure shows a typical experiment representative of three independent experiments giving similar results.

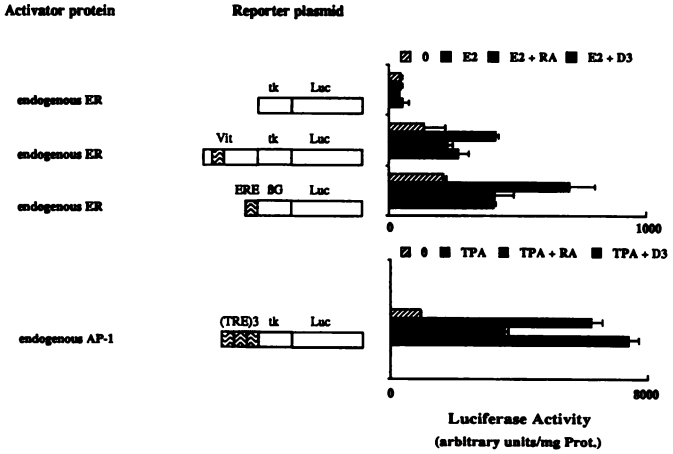

Fig. 5. Effect of RA and Vit D_3 on various reporter systems. MCF-7 cells plated in 6-multiwell dishes were transiently transfected with the indicated reporter plasmid (500 ng/well). Cells were treated for 24 h with the positive effector (E2 10 nM or TPA 20 nM) in the presence or absence of RA (200 nM) or Vit D_3 (100 nM); control cells (0) received the vehicle (ethanol or dimethyl sulfoxide). At the end of incubation, the luciferase activity was determined, and the results are expressed as arbitrary units/mg protein ± SE.

inhibited ER binding to the ERE. In addition, this inhibition was reversed by increasing the ERE concentration, suggesting a competitive inhibition. However, this does not prove that antiestrogenic effects of RA and Vit D_3 involve competitive inhibition by RAR or VDR as homodimers since such extracts may contain auxiliary protein(s) able to interact with the expressed RAR or VDR to inhibit ER/ERE binding.

As a control, the ER dissociation constant for the biotinylated ERE performed in the presence of WCE of untransfected COS cells was identical to that reported previously (43). Moreover, ER binding to the biotinylated ERE was displaced by adding excess unbiotinylated ERE, and it was unaffected in the presence of unspecific DNA (salmon sperm; data not shown).

We then analyzed, by transient transfection of MCF-7 cells, the specificity of the inhibitory effects of RA and Vit D_3 on the estrogen regulatory part. As shown in Fig. 5, the results strongly suggested that a single ERE was sufficient to maintain the antagonistic effect of Vit D_3 and RA because, regardless of the promoter part (Vit-tk or ERE-βG), RA and Vit D_3 inhibited estrogen-induced luciferase. We then tested the effect of RA and Vit D_3 on various plasmid constructions in which either the ERE part was missing (tk-Luc) or replaced by other regulatory parts [$(TRE)_3$-tk-Luc or $(RARE)_3$-tk-Luc]. No effects of RA or Vit D_3 were observed on the parental tk-Luc plasmid (Fig. 5). There was no effect of Vit D_3 on TPA- [transfection with $(TRE)_3$-tk-Luc; Fig. 5] or RA-induced luciferase [transfection with $(RARE)_3$-tk-Luc; data not shown]. RA induced luciferase expression through $(RARE)_3$-tk-Luc, while it inhibited TPA-induced luciferase through $(TRE)_3$-tk-Luc; this result is in agreement with the well-known inhibitory effect of RA on AP-1 regulated genes (45). Taken together, these results show that the RA and Vit D_3 inhibitory effects on Vit-tk-Luc or ERE-βG-Luc were specific to an estrogenic regulatory function.

What is the Molecular Mechanism of Antagonistic Action of RA and Vit D_3?

We investigated the mechanism of inhibitory action of RA and Vit D_3 by transient transfection experiments performed with chimeric receptor constructions and appropriate reporter plasmids (Fig. 6). To analyze the role of the ER DNA binding domain (ER-C) and ERE in this inhibition, we used the chimeric Gal-ER receptor in which the DNA binding domain of the yeast transcription factor GAL4 is fused to the hormone binding, transactivation (TAF-2), and dimerization domains of ER (ER-DEF). As shown in Fig. 6, the antagonistic effect of RA disappeared while that of Vit D_3 was maintained. This suggested that the specific DEF domain of ER was not required to obtain an inhibitory effect of RA while it was required to obtain that of Vit D_3. We were thus interested to ascertain (*a*) what part of ER was required for RA inhibition and (*b*) whether the presence of the ER-DEF was sufficient to obtain Vit D_3-mediated inhibition. To answer these questions, we used the chimeric receptor GR-ERcass containing the NH_2-terminal A/B and COOH-terminal DEF domains of GR and ER-C. In fact, the inhibitory effect of Vit D_3 was greatly decreased in the absence of ER-DEF, while the RA antagonist effect was maintained (Fig. 6). On the contrary, by testing RA and Vit D_3 effects on the chimeric receptor ER-GRcass containing the NH_2-terminal AB and COOH-terminal DEF domains of ER and DNA binding domain of GR (GR-C), the inhibitory effect of RA was greatly decreased while the inhibitory effect of Vit D_3 was maintained. Taken together, these results show that ER-DEF is able to confer a sensitivity to the Vit D_3 inhibition, while ER-C is able to confer a sensitivity to the RA inhibition.

We then wondered whether RXR contributes to the antagonistic effect of RA and Vit D_3 since RXR is a coregulator protein for RAR and VDR by increasing their specific DNA binding and transactivity. MCF-7 cells were thus transiently transfected with RAR, RXR, or VDR to analyze their effects on RA- or Vit D_3-mediated inhibition. As shown in Fig. 7*A*, the addition of RAR or RXR alone did not modulate the antagonistic effect of RA, while the addition of RAR combined with RXR markedly increased the antagonistic effect of RA. As shown in Fig. 7*B*, the addition of VDR slightly increased the antagonistic effect of Vit D_3, while cotransfection of RXR with or without VDR did not modulate the inhibitory effect of Vit D_3.

DISCUSSION

In ER-positive breast carcinoma cells (like MCF-7), cellular proliferation is essentially under estrogenic control, and antiestrogenic compounds are able to suppress this estrogen-induced proliferation (46). Antiestrogens are also widely used in clinics for antihormonal therapy of estrogen-dependent breast cancer, although they are not fully efficient in all cases. The growth inhibitory effect of RA and Vit D_3 on hormone-dependent breast tumors is well documented (47–53). Since nuclear receptors for RA and Vit D_3 belong to the same subfamily as the ER, we investigated potential cross-talk between estrogenic action and RARs or VDR in these cells. We report that RA and Vit D_3 behaved as antiestrogenic compounds since they were able to

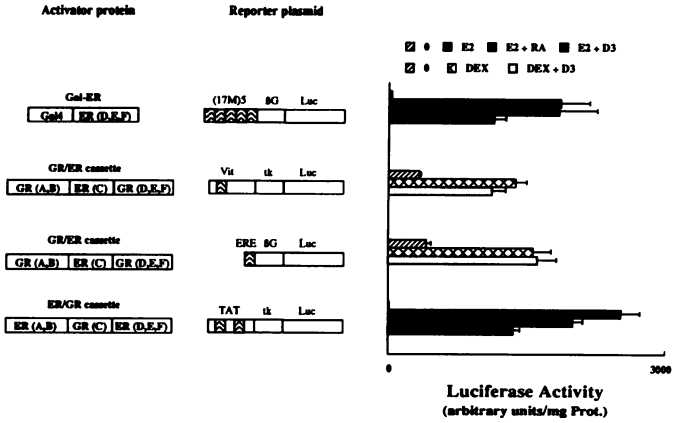

Fig. 6. Effect of RA and Vit D_3 on various chimeric expression systems. MCF-7 cells plated in 6-multiwell dishes were transiently cotransfected with the indicated reporter plasmid (800 ng/well) and with expression plasmids of various chimeric receptors (200 ng/well). As described, transiently transfected cells were treated for 24 h with the positive effector (E2 10 nM or dexamethasone 100 nM) in the presence or absence of RA (200 nM) or Vit D_3 (100 nM); control cells (0) received the vehicle (ethanol). At the end of incubation, the luciferase activity was determined, and the results are expressed as arbitrary units/mg protein ± SE. A typical experiment using the GR-ERcass and Vit-tk-luc as reporter was also performed in MCF-7 cells with the same experimental conditions as above and gave the following results (mean of arbitrary units/mg protein): control, 86; E2, 150; RA, 35; RA + E2, 70; Vit D_3, 33; and Vit D_3 + E2, 132.

inhibit both estrogen-dependent growth of MCF-7 cells and estrogen-dependent gene transcription.

Our results on MCF-7 cell growth are in agreement with previous studies showing that the effect of RA and Vit D_3 is additive to that of the classical antiestrogen OH Tam to produce an antiproliferative effect (54). Because the estrogen-dependent growth of MCF-7 cells is a complex response involving many regulatory pathways and a network of multiregulated genes, we focused on a more estrogen-specific gene regulation. We then showed that RA and Vit D_3 inhibited natural endogenous estrogenic response, such as pS2 mRNA induction, and an estrogenic response from a chimeric gene in MVLN cells, a derived MCF-7 cell line. Similar results were reported previously with retinoic acid on pS2 mRNA in MCF-7 cells and on PR expression in T-47D cells (55, 56). In MVLN cells, RA and Vit D_3 were unable to bind ER, they did not modulate the binding parameters of estradiol to its receptor, and their inhibitory effects could not be reversed by increasing estradiol concentration. This clearly distinguished the inhibitory effects from that of classical antiestrogens (acting by competition with estradiol to bind the ER). Interestingly, as for the estrogen-dependent growth of MCF-7 cells, the effect of RA and Vit D_3 was additive to that of OH Tam for inhibiting the estrogen-dependent gene expression of luciferase in MVLN cells.

The antagonistic effect of RA and Vit D_3 on estrogen-induced gene expression was further investigated at the ERE level. ABCD analysis showed that crude cell extracts containing RAR or VDR competitively inhibited ER binding to ERE, but it is not clear whether this inhibitory effect occurred by direct competition between RAR or VDR with ER for the ERE or by heterodimerization with a partner protein. Nonetheless, this result suggested that RARs and VDR may play a part in impairing the binding of the ER to the ERE. By transient transfection experiments using different regulatory parts of the chimeric response gene, we demonstrated that the inhibitory effect was specific to the estrogenic regulatory function. This may occur through various mechanisms.

In the ER/TR subfamily of nuclear receptors, several types of interactions occur providing a diversity of hormone responses; the dimerization domain structure of this group of receptors favors heterologous protein-protein interactions, while their differentially organized response elements multiply their selectivity parameters (11, 57, 58). Negative transcriptional effects may be exerted by these receptors through several possible mechanisms (59, 60). One involves competition between receptors acting on the same DNA sequence. Thus, Lipkin *et al.* (61) showed that the RAR binding to a negative RARE closely related to an ERE needs the presence of other proteins. Similarly, Glass *et al.* (25) showed that the TR competed with the ER to bind the palindromic ERE and inhibited the transcriptional activity of the latter. Another type of negative regulation occurs via protein-protein interactions; this may result from the sequestration of nonspecific transcription factors or squelching (62) or through formation of nonfunctional heterodimeric complexes (45, 63). These complexes are generally not able to bind DNA or if they do, there is no transcriptional activity. For example, TR forms heterodimers with RAR which increase the binding of RAR to a subset of thyroid hormone response elements. Interestingly, these heterodimers activate transcription through a palindromic thyroid hormone response element but have a negative effect on the response element derived from the α myosin heavy chain.

We used several chimeric expression systems to determine by which of these mechanisms RA and Vit D_3 inhibited estrogenic action. We showed that RA needed an estrogenic pathway involving ER-C and ERE to exert its antagonistic effect since no inhibition was observed using the Gal-ER chimeric construction. The slight inhibition of RA observed with a chimeric system involving ER-GRcass and the reporter TAT-tk-Luc suggest that TAF-1 of ER could slightly contribute to the antagonistic effect of RA; there is probably no contribution of the GR part since, in stably transfected HeLa cells expressing luciferase in a glucocorticoid-dependent manner, several retinoid analogues were tested, and no inhibitory effect was obtained on this glucocorticoid response (data not shown). Our results show that the Vit D_3 antagonistic effect was observed by using chimeric receptors containing the DEF domain of ER fused to various DNA binding domains, thus suggesting a primordial role of the DEF domain of ER.

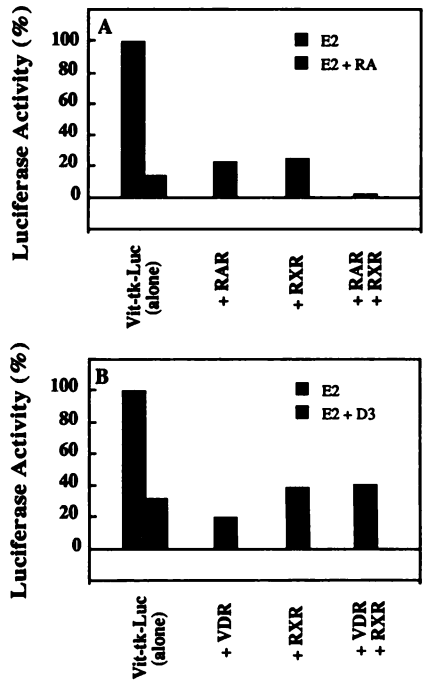

Fig. 7. Effect of RAR, RXR, and VDR on the inhibitory effect of RA and Vit D_3 on a gene transcriptionally controlled by the ER. MCF-7 cells, plated in 6-multiwell dishes, were transiently transfected with Vit-tk-Luc (800 ng/well) alone or in combination with expression plasmids RAR, RXR, and VDR (200 ng/well). As described, transiently transfected cells were treated for 24 h with 10 nM E2 alone or with the addition of 200 nM RA *(A)* or 100 nM Vit D_3 *(B)*. At the end of incubation, the luciferase activity was determined, and the results are expressed as the mean percentage taking the luciferase activity induced by 10 nM estradiol as 100%.

RA and Vit D_3 thus differed from each other by the target responsible for the inhibitory effect of the estrogen action. For Vit D_3, its antagonistic effect would be found especially at the trans-acting protein ER level. For RA, the antagonistic effect would be found rather at the cis-acting sequence ERE.

Since RXR has been described as the coregulator of RAR and VDR by forming heterodimers which bind their target DNA more efficiently, we analyzed, by transient transfection experiments, whether RXR could modulate the inhibitory effect of RA and Vit D_3. The antagonistic effect of RA was markedly increased by adding RAR combined with RXR, indicating that RAR and RXR can interfere with ER at the ERE level. This conclusion is in agreement with results obtained with oxytocin (61) or vitellogenin A2 and B1 ERE (64, 65). Involvement of RXR in the RA-dependent inhibition of an estrogen response was further supported by the fact that addition of VDR partially reversed the antiestrogenic effect of RA (data not shown); this could correspond to the titration of RXR by VDR in the same way as reported recently for the thyroid hormone receptor (66). Moreover, 9-*cis*-RA is also able to inhibit luciferase expressed in an estrogenic-dependent manner (data not shown).These different points indicated that RAR and RXR can interfere with ER at the ERE level, but we cannot conclude whether the inhibition mechanism involves heterodimerization of RAR with RXR and/or with other auxiliary proteins.

By contrast, we did not observe any modulation of the antagonistic effect of Vit D_3 by RXR, suggesting that the heterodimer VDR/RXR is not responsible for the antiestrogenic effect of Vit D_3. However, addition of VDR slightly increased the antagonist effect of Vit D_3, suggesting that VDR would mediate the antagonistic effect of Vit D_3. This conclusion is also supported by the fact that several potent analogues of Vit D_3 able to bind VDR lead to a potent antagonistic effect on a gene transcriptionally controlled by ER in MVLN cells, while Vit D_3 analogues unable to bind VDR were inefficient (data not shown). Since we showed that the DEF domains of ER (ligand binding, dimerization, and ligand-dependent transactivating domains) were required for the inhibitory effect of Vit D_3, there could be either a direct or an indirect interaction of VDR with the ER. In both cases, we cannot forget that, in the ABCD assay, ER remained able to bind to the ERE since the inhibitory effect decreased by increasing the ERE concentration. In the case of direct inhibition, there would be an interaction between the ER and VDR, leading to the formation of ER/VDR heterodimers which bind ERE with lower affinity than ER homodimers. Whereas in the case of indirect inhibition, VDR would titrate a protein involved in the binding of ER to ERE. In this context, several proteins have been described as being necessary for ER binding to its response element (67, 68). In the same way, we do not exclude the idea that the antagonistic effect of RA would also involve an indirect interaction with another protein.

Both RA and Vit D_3 are frequently used in clinical or laboratory cancer studies and have been shown to induce differentiation and inhibit proliferation of various cancer cells including breast tumors. Differentiation therapy is therefore an appealing alternative, particularly when classical therapies fail. However, the major drawback to the use of RA or Vit D_3 as therapeutic agents is their diverse toxic effects at pharmacological doses. For this reason, discovery of more specific actions for potential combinatorial or interchanging therapy may be a means to enhance their use. Taken together, our data suggest that the antiproliferative effect of RA and Vit D_3 may be partially explained by their antagonistic effects on estrogenic action. This effect differed from and was additive to that of OH Tam, which is an estrogen antagonist and the natural metabolite of tamoxifen, a widely used therapeutic agent in breast cancer. Further analysis of the inhibitory mechanisms and determination of RAR and VDR ligands with lower intrinsic but higher antiestrogenic activities will be the future challenge of this study.

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