1,25 $(OH)_2$ Vitamin D_3 and Retinoic Acid Antagonize Endothelin-stimulated Hypertrophy of Neonatal Rat Cardiac Myocytes

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Abstract

1,25 $(OH)_2$ Vitamin D_3 (VD_3) and retinoic acid (RA) function as ligands for nuclear receptors which regulate transcription. Though the cardiovascular system is not thought to represent a classical target for these ligands, it is clear that both cardiac myocytes and vascular smooth muscle cells respond to these agents with changes in growth characteristics and gene expression. In this study we demonstrate that each of these ligands suppresses many of the phenotypic correlates of endothelin-induced hypertrophy in a cultured neonatal rat cardiac ventriculocyte model. Each of these agents reduced endothelin-stimulated ANP secretion in a dose-dependent fashion and the two in combination proved to be more effective than either agent used alone (VD_3: 49%; RA: 52%; VD_3 + RA: 80% inhibition). RA, at concentrations known to activate the retinoid X receptor, and, to a lesser extent, VD_3 effected a reduction in atrial natriuretic peptide, brain natriuretic peptide, and α-skeletal actin mRNA levels. Similar inhibition (VD_3: 30%; RA: 33%; VD_3 + RA: 59% inhibition) was demonstrated when cells transfected with reporter constructs harboring the relevant promoter sequences were treated with VD_3 and/or RA for 48 h. These effects were not accompanied by alterations in endothelin-induced c-*fos*, c-*jun*, or c-*myc* gene expression, suggesting either that the inhibitory locus responsible for the reduction in the mRNA levels lies distal to the activation of the immediate early gene response or that the two are not mechanistically coupled. Both VD_3 and RA also reduced [^{3}H]leucine incorporation (VD_3: 30%; RA: 33%; VD_3 + RA: 45% inhibition) in endothelin-stimulated ventriculocytes and, once again, the combination of the two was more effective than either agent used in isolation. Finally, 1,25 $(OH)_2$ vitamin D_3 abrogated the increase in cell size seen after endothelin treatment. These findings suggest that the liganded vitamin D and retinoid receptors are capable of modulating the hypertrophic process in vitro and that agents acting through these or similar signaling pathways may be of value in probing the molecular mechanisms underlying hypertrophy. (*J. Clin. Invest.* 1996. 97:1577–1588.) Key words: cardiac hypertrophy • gene regulation • atrial natriuretic peptide • brain natriuretic peptide • α-skeletal actin

Introduction

The seco-steroid 1,25 $(OH)_2$ vitamin D_3 is a polar metabolite of vitamin D which binds with high affinity to the vitamin D receptor (VDR)[1] in target cells. This liganded receptor interacts with specific recognition elements (consensus sequence represented by AGGTCANNNAGGTCA) (1) present in the regulatory regions of target genes and, through an as yet undefined mechanism, triggers an increase or reduction in transcriptional activity. This activity may be amplified through association of the liganded VDR with a second regulatory protein, the retinoid X receptor (RXR). This association produces a heterodimeric complex which binds the recognition element more avidly and regulates transcription with greater efficiency (2).

Though not considered a traditional target for 1,25 $(OH)_2$ vitamin D_3, the cardiovascular system is sensitive to the regulatory activity of this hormone. High-affinity receptors for 1,25 $(OH)_2$ vitamin D_3 have been identified in both myocardial (3) and vascular smooth muscle (4) cells. In the cardiac myocyte vitamin D has been linked to enhanced calcium transport across the plasma membrane (5). In addition Weishaar and Simpson have shown that 1,25 $(OH)_2$ vitamin D_3 deficiency is associated with aberrant cardiac contractility and hypertrophy in a rodent model (6). The same group has identified changes in cardiac morphology, largely confined to the interstitial compartment (7), as well as changes in the myosin isozyme profile (8) in states of vitamin D deficiency.

Retinoic acid (RA), another nuclear hormone receptor ligand, has also been shown to have important effects on the heart. Mice homozygous for deletion of the RXRα gene manifest developmental abnormalities in the cardiovascular system (9, 10) with thin-walled ventricles reminiscent of atrial myocardium (11). These animals die in utero, presumably due to congestive cardiac failure. Superimposing deletions in the RA receptors (i.e., RARα or RARγ) appears to amplify some features of the RXRα −/− phenotype (10), suggesting that retinoid receptor–signaled activity may converge at key points in embryogenesis to promote normal development of the cardiovascular system.

Because adult cardiac myocytes lack mitotic capability, they respond to growth stimuli largely through hypertrophy of existing myocardial cells rather than through an increase in cell number (i.e., hyperplasia). In vivo hypertrophy is activated in

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CAT, chloramphenicol acetyltransferase; ET, endothelin; FS, flanking sequence; OCT, oxacalcitriol; RA, retinoic acid; RAR, RA receptors; RXR, retinoid X receptor; TK, thymidine kinase; VDR, vitamin D receptor; VDRE, vitamin D response element.

situations associated with hemodynamic overload. This may arise, in part, from the mechanical tension placed on the individual cardiac myocytes and partially from activation of reflex neuroendocrine activity (e.g. α-adrenergic activity and renin-angiotensin system) which accompanies overload (12, 13). In vitro models of hypertrophy which mimic the in vivo paradigm with absolute fidelity are lacking; however, many studies have focused on a model which closely resembles hypertrophy in cultured neonatal rat ventricular myocytes (14). These cells respond to a variety of stimuli, including α-adrenergic agonists, endothelin (ET), angiotensin, growth factors, and mechanical strain with an increase in macromolecular synthesis and changes in cell morphology which resemble those seen with cardiac hypertrophy (15–19). ET, in particular, has attracted considerable interest as a potential autocrine or paracrine modulator of hypertrophy. ET gene expression is activated in cardiac myocytes by stimuli which promote hypertrophy (20, 21). Furthermore, ET receptor antagonists (22) have been shown to reverse hypertrophy in aortic-banded rats in vivo. Hypertrophic stimuli provoke a predictable and sequential activation of gene expression which mimics that seen with in vivo hypertrophy (23). The first genes to be activated are those of the immediate early gene family (e.g., c-*jun*, c-*fos*, c-*myc*, and egr-1), a group of protooncogene products which function predominantly as transcription factors in the cell nucleus. This is followed by the activation of a second group of genes collectively referred to as the embryonic repertoire or fetal gene program. This group includes the α-skeletal actin, β-myosin heavy chain, and the atrial natriuretic peptide (ANP) genes. As a group these genes are typically expressed in the fetal and early neonatal ventricle. Expression decreases in the weeks after birth and remains quiescent in the adult myocardium unless the latter is subjected to a hemodynamic load capable of generating hypertrophy. In the latter circumstance there is a reactivation of the fetal program which persists for varying intervals thereafter. ANP and β-MHC gene expression tends to remain elevated for extended periods of time while α-skeletal actin expression returns to basal levels within a few days despite continued application of the stimulus. A third group of genes is activated later in the hypertrophic process. This group, which includes the cardiac actin and the myosin light chain-2 genes, is in large part responsible for providing the molecular substrate for enlargement of the sarcomeric structure and hypertrophy of the myocardial cell. The relationship which exists among these three groups of genes is based largely on their kinetics of appearance, although there are data suggesting that increases in immediate early gene expression may be tied mechanistically to activation of the fetal gene program (24, 25).

Previously we have shown that 1,25 $(OH)_2$ vitamin D_3 negatively regulates expression of the endogenous ANP gene (26) as well as a transfected human (h) ANP-CAT reporter in cultured rat atriocytes (27). This inhibition was dose dependent with regard to both ligand and receptor and appeared to be amplified in the presence of cotransfected RXR. Since ANP gene expression is viewed as one of the earliest and most reliable markers of hypertrophy in the cardiac ventriculocyte, we have examined the ability of these two ligand receptor systems to antagonize ET-induced hypertrophy in these cells. Our findings demonstrate that the hypertrophic process, assessed through measurement of a number of conventional biochemical and morphological markers, is suppressed in the presence of these ligands.

Methods

Materials. [^{3}H]Leucine, [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{3}H]acetyl coenzyme A were purchased from DuPont/NEN Research Products (Boston, MA). 1,25 $(OH)_2$ Vitamin D_3 was obtained from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Oxacalcitriol (OCT) was generously provided by Chugai Pharmaceutical Co., Ltd. (Shizuoka, Japan) and 9-*cis* RA by Dr. Arthur A. Levin of Hoffman-LaRoche (Nutley, NJ). All-*trans* RA was purchased from Sigma Chemical Co. (St. Louis, MO). ET-1 was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Fluorescein-tagged phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents were purchased from standard commercial suppliers.

Cell preparation. Ventricular myocytes were isolated from the lower two-thirds of 1-d-old neonatal rat hearts by alternate cycles of trypsin digestion and mechanical disruption as previously described (28). Myocytes were separated from mesenchymal cells (primarily fibroblasts) using a 30-min preplating step which fostered selective adherence of fibroblasts but not myocytes to the culture surface. Fibroblast cultures were propagated from these initial preplates in serum-containing medium and expanded through a single replating (split 1:4) as described previously (28). Myocytes were either plated directly in plastic culture dishes or used for electroporation (see below) before plating. For [^{3}H]leucine incorporation, ANP secretion, and mRNA measurements, cells were cultured in Dulbecco's modified Eagle's medium (DME H-21) containing 10% bovine calf serum (EC) (Gemini Bioproducts, Calabasas, CA) and bromodeoxyuridine (0.03 mg/ml) for 3 d to suppress fibroblast proliferation and then changed to fresh serum-containing medium without bromodeoxyuridine for an additional day. Before the initiation of each experiment, cultures were placed in serum-free medium (29), and all subsequent experimental manipulations were carried out in this medium. Transfected cells were cultured in DME H-21 containing 10% EC for 24 h before switching to the serum-free medium. Specific chemical additives or agonists were diluted 1:1,000 from stock solutions into serum-free medium; similar concentrations of ligand-free vehicle were without effect in these cultures.

Protein synthesis. Protein synthesis in the cultured neonatal rat ventricular myocytes was assessed by measuring [^{3}H]leucine incorporation into acid-insoluble cellular material. Ventricular myocytes were plated in 24-well dishes at a density of 1×10^5 cells/well. After changing to serum-free medium for 24 h, cells were treated with different concentrations of 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA for defined periods of time. Where indicated, ET (10^{-7} M) was added for the final 24 h of culture. For 4 h before collection cells were pulsed with [^{3}H]leucine in leucine-free medium containing the same additives. Cells were washed twice with cold PBS, once with 10% TCA, and extracted with 10% TCA at 4°C for 30 min. Cell residues were rinsed in 95% ethanol, solubilized in 0.25 N NaOH at 4°C for 2 h, and then neutralized with 2.5 M HCl plus 1 M Tris-HCl (pH 7.5). The radioactivity was determined by liquid scintillation spectrophotometry.

Actin staining. Ventricular myocytes were cultured in 8-chamber culture slides (Nunc Inc., Naperville, IL) at a density of 4×10^4 cells/chamber in 10% EC for 2 d, and then changed to serum-free DME containing 10^{-8} M 1,25 $(OH)_2$ vitamin D_3 or vehicle alone for 48 h. ET (10^{-7} M) was included in the relevant samples for last 24 h of the incubation. The medium was discarded; the cell layer was rinsed twice with PBS, fixed with 3.7% formaldehyde for 10 min at room temperature, rinsed twice again with PBS, extracted with acetone for 5 min at −20°C, air dried, and exposed to the fluorescein-labeled phalloidin probe, diluted as recommended by the manufacturer, for 20 min at room temperature. After two rinses with PBS, the cell layer was covered with PBS/glycerol (1:1) solution, protected with a coverslip, and stored in the dark at 4°C. Photomicrographs were taken with a Leitz epifluorescence microscope. Measurements of two-dimensional cell surface areas were made on a Macintosh Quadra 660AV computer using the public domain NIH Image Program (developed at the U.S.

National Institutes of Health and available from the Internet by anonymous FTP from *zippy.nimh.nih.gov.* or on a floppy disk from the National Technical Information Service, Springfield, VA, part number PB 95-500195GEI).

Radioimmunoassay for ANP. Ventricular myocytes were plated in 24-well dishes at a density of 1×10^5 cells/well and cultured as described above. 24 h before treatment, the cultures were shifted into serum-free media and maintained under these conditions throughout the remainder of the experiment. Cells were treated with 1,25 $(OH)_2$ vitamin D_3, all-*trans* RA and/or ET as described above (see *Protein synthesis*). Aliquots of media from the final 24-h culture period were collected, centrifuged to remove cellular debris, and frozen at −20°C until assayed. Radioimmunoassay was carried out using a rabbit anti–rat ANP antibody and rat $ANP_{1–28}$ as the standard and radiolabeled tracer, as described previously (30).

RNA isolation and Northern blot analysis. Ventricular myocytes were plated in 10-cm dishes and cultured and treated with 1,25 $(OH)_2$ vitamin D_3 and/or RA as described above. Total RNA was extracted from the cells by the guanidinium thiocyanate–CsCl method (31). 15 μg of RNA was size fractionated on a 2.2% formaldehyde/1% agarose gel, transferred to nitrocellulose filters, and hybridized either with an 840-bp rat ANP cDNA probe (30) isolated as a HindIII-EcoRI fragment, or a 640-bp EcoRI fragment of the rat brain natriuretic peptide (BNP) cDNA (32). Each of these probes was labeled with [α-^{32}P]dCTP using the random primer method. A 20-bp α-skeletal actin antisense oligonucleotide (5′ GCAACCATAGCACGATGGTC 3′) (33) was labeled with [γ-^{32}P]ATP using polynucleotide kinase. Individual blots were subsequently washed and reprobed with a 1.15-kb BamHI-EcoRI fragment of 18S ribosomal cDNA to permit normalization of blots for differences in RNA loading and/or transfer to the filter. Autoradiography was performed with an intensifying screen at −70°C for 3 h to 1 d. Autoradiographic signals were quantified by laser densitometry.

Plasmid constructions. The reporter plasmid −1150 hANP CAT containing 1,150 bp of 5′-flanking sequence (FS) from the human ANP gene linked to bacterial chloramphenicol acetyltransferase (CAT) coding sequence has been described previously (34). A second reporter plasmid, −1173 hBNP CAT, containing 1,173 bp of 5′-FS from the human BNP gene linked to CAT coding sequence, was generated from a plasmid (35) containing ~ 3,200 bp of hBNP genomic sequence, by PCR using an upstream sense oligonucleotide (5′ CCTCTAGACCAGGCTGGAGTGCAGTGGCG 3′) which incorporated an XbaI site at its 5′ terminus and a downstream antisense oligonucleotide (5′ CCAAGCTTGGGACTGCGGAGGCTG 3′) which incorporated a HindIII site at its 5′ terminus. A PCR product of the appropriate size was restricted with XbaI and HindIII and subcloned into compatible sites of pSVoLCAT (24, 36) Another reporter plasmid, −1400 α-skeletal actin CAT, containing 1,400 bp of 5′-FS from the mouse α-skeletal actin gene promoter linked to CAT, was kindly provided by P. Simpson (37). Both −109TKCAT (28) and DR3CAT (27) have been described previously. The latter contains a direct repeat vitamin D response element (VDRE) with three nucleotide spacing (5′-AGGTCAaggAGGTCA-3′) immediately upstream from the thymidine kinase (TK) promoter. The expression vectors were generated as follows. The full-length cDNA for the human VDR was generously provided by Dr. A. Baker (38). A 2.0-kb EcoRI fragment containing the entire coding sequence for the receptor was subcloned into pSV_L; this latter vector allows expression of receptor coding sequence from the SV40 promoter. The expression vector, pRShRXRα, encoding the human RXR has been described (39), it places 1,832 bp of hRXRα cDNA under the control of the RSV promoter. pRShRARα (40) places 1,943 bp of hRARα cDNA downstream from the RSV promoter.

DNA transfection and CAT assay. Ventricular myocytes were transfected with 20 μg of hANP-, hBNP-, or α-skeletal actin CAT together with 5–10 μg VDR, RAR, and/or RXR expression vector(s) where indicated. Increasing vector DNA to as much as 15 μg per transfection did not amplify the observed effect beyond that seen at the 5-μg level. The total amount of transfected DNA was adjusted with PUC18. Transient transfection was achieved by electroporation (Gene Pulsar; Bio-Rad Laboratories, Richmond, CA) using 280 V at 250 μF, optimal conditions which were derived empirically. 10×10^6 cells were used for each transfection group. After transfection, cells were plated in 6-well dishes at a density of 3×10^6 cells/well in 10% EC/DME. Medium was changed at 24 h to serum-free DME containing the appropriate reagent(s) and the incubation was continued for an additional 48 h. Cells were then harvested and lysed in 250 mM Tris/0.1% Triton X-100. Protein concentration of each cell extract was measured using the Coomassie protein reagent (Pierce, Rockford, IL). Cell lysates were processed for CAT assay (50–100 μg cellular protein was used for each group); a mock reaction containing no protein was included in each assay to establish a background activity which, in turn, was subtracted from each experimental value. To ensure reproducibility, experiments were repeated three to six times, using at least three different plasmid DNA preparations.

Statistical analysis. Unless stated otherwise, statistical differences were evaluated by one-way ANOVA with the Newman-Keul's test for significance.

Results

Since ANP gene expression and secretion of the encoded peptide product is purported to be one of the most sensitive markers of the hypertrophic process, we examined the effect of 1,25 $(OH)_2$ vitamin D_3 and RA on ET-stimulated secretion of this peptide from cultured neonatal rat ventriculocyte cultures. Neither all-*trans* RA, at concentrations known to occupy the

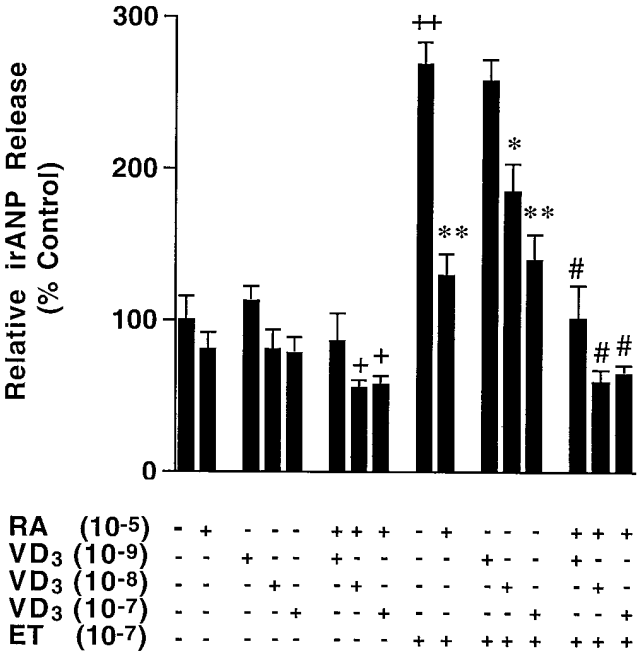

Figure 1. Effect of 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA on ET-stimulated irANP secretion from cultured ventricular myocytes. Cells were treated with 1,25 $(OH)_2$ vitamin D_3 (10^{-8} M) and/or all-*trans* RA (10^{-5} M) for 48 h in serum-free medium. Where indicated, 10^{-7} M ET was added to the medium for the final 24 h. Medium was taken for irANP radioimmunoassay. Values are expressed relative to measurements made in untreated control group. Data represent means±SD from three different experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ET 10^{-7} M; $^{\#}P < 0.01$ vs. corresponding ET + VD_3 group.

RXR receptor (41), nor 1,25 $(OH)_2$ vitamin D_3 had a significant effect on basal irANP secretion when used alone; however, when used together [i.e., maximal concentration of RA and increasing concentrations of 1,25 $(OH)_2$ vitamin D_3] there was a significant reduction in hormone release which was maximal at 10^{-8} M 1,25 $(OH)_2$ vitamin D_3 (Fig. 1). As expected, ET effected a significant increase in irANP release. This increase was reversed in a dose-dependent fashion by 1,25 $(OH)_2$ vitamin D_3. Once again, the inclusion of RA amplified the 1,25 $(OH)_2$ vitamin D_3 effect, reducing secretory activity to levels well below those seen in the basal state and similar to those seen with 1,25 $(OH)_2$ vitamin D_3 and RA alone.

The effect on irANP release was at least partially related to effects on hormone synthesis. As shown in Fig. 2, 1,25 $(OH)_2$ vitamin D_3 effected a modest reduction in both basal and ET-stimulated ANP mRNA levels in these cultures. All-*trans* RA, on the other hand, had little effect on basal ANP gene expression yet reduced ET-stimulated ANP transcript levels by $> 50\%$. The combination of both agents together was not more efficacious than RA alone.

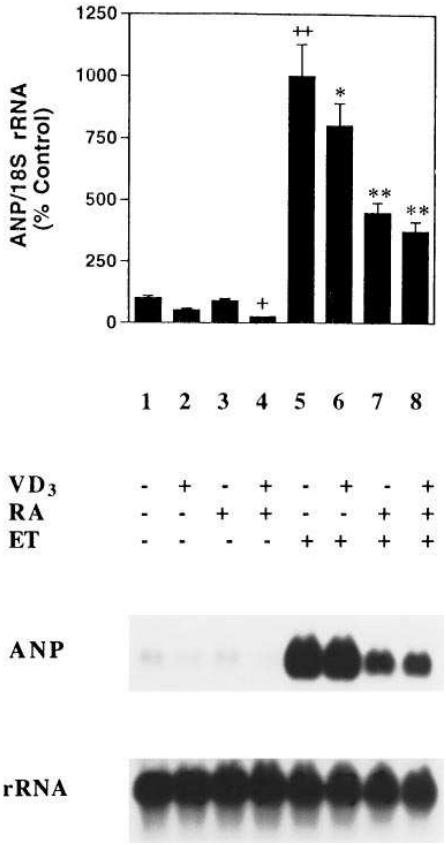

Figure 2. Effect of 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA on ET-stimulated expression of ANP gene in cultured ventricular myocytes. Cells were treated with 1,25 $(OH)_2$ vitamin D_3 (10^{-8} M) or all-*trans* RA (10^{-5} M) for 48 h in serum-free medium. Where indicated, 10^{-7} M ET was added to the medium for the final 24 h. Cells were then harvested and total RNA was prepared. 15 μg RNA from each group was size fractionated, transferred to a nitrocellulose filter, and sequentially blot hybridized with radiolabeled cDNAs for ANP and 18S ribosomal RNA. Histogram depicts average values of densitometric scans obtained from three experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ET group.

As discussed above, expression of a number of other genes is activated as a consequence of hypertrophy. Included within this group are α-skeletal actin, an actin isoform which is preferentially expressed in the fetal myocardium, and BNP (42). Therefore, it was of interest to determine whether the pronounced effects on ANP gene expression noted above could be extrapolated to these other markers of hypertrophy. As shown in Fig. 3, RA and to a lesser extent 1,25 $(OH)_2$ vitamin D_3 were effective inhibitors of ET-dependent increments in BNP mRNA levels. The modest increments in basal transcript levels seen in this autoradiograph after 1,25 $(OH)_2$ vitamin D_3 or RA treatment were not reproducible in other experiments (Fig. 3, bar graph at right). The combination of 1,25 $(OH)_2$ vitamin D_3 and RA was only slightly more effective than RA alone ($P < 0.05$) in suppressing BNP gene expression. In a similar fashion, basal α-skeletal actin mRNA levels were only slightly affected by 1,25 $(OH)_2$ vitamin D_3 or RA treatment while ET provoked a large increase in expression (Fig. 3). Both RA and 1,25 $(OH)_2$ vitamin D_3 fostered a truncation of this ET effect and, in this case, the combination was clearly more effective than either agent used alone ($P < 0.01$).

1,25 $(OH)_2$ Vitamin D_3 is a calcitropic hormone which fosters calcium transport across the gut mucosa. It is also known to alter calcium flux across the sarcolemmal membrane of the cardiac myocyte (5) raising the possibility that the inhibitory activity noted above could reflect a secondary effect of accelerated calcium transport into or out of the cardiac myocyte. To explore this possibility, we used OCT, a nonhypercalcemic analogue of 1,25 $(OH)_2$ vitamin D_3 (43), in an effort to determine whether the inhibitory effect would be preserved in the absence of these calcium-transporting properties. As shown in Fig. 3, ET-stimulated α-skeletal actin gene expression was reduced equivalently by 1,25 $(OH)_2$ vitamin D_3 and OCT, and these individual effects were amplified further by the addition of all-*trans* RA. Similar levels of suppression of ET-stimulated ANP and BNP mRNA levels were seen with the OCT/RA vs. 1,25 $(OH)_2$ vitamin D_3/RA combination (data not shown). This suggests that the inhibitory effect of 1,25 $(OH)_2$ vitamin D_3 on cardiac gene expression can be dissociated from its calcium mobilizing activity.

As mentioned above, a number of protooncogene products are also increased as a consequence of hypertrophy in the cardiac myocyte (23). These immediate early gene products are stimulated within a manner of minutes after application of a variety of hypertrophic stimuli and may be linked to the activation of downstream events (e.g., transcription of the fetal gene program) in the hypertrophic cascade (24, 25). To probe the possible involvement of the inhibitory agonists in perturbing very early responses to hypertrophic stimuli, we investigated the effect of 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA on ET-stimulated immediate early gene expression. As shown in Fig. 4, ET increased levels of the c-*fos*, c-*jun*, and c-*myc* transcripts severalfold. In no instance did 1,25 $(OH)_2$ vitamin D_3 or RA, alone or in combination, have any effect on the expression of these protooncogene products. This argues for the selectivity of the inhibitory activity seen with the marker gene products described above and implies that reduction in immediate early gene expression cannot be invoked to account for the truncation of the ET-dependent activity.

In an effort to explore the mechanism(s) underlying the reduction in steady state levels of the ANP mRNA, we introduced a chimeric hANP-CAT reporter into cardiac ventriculo-

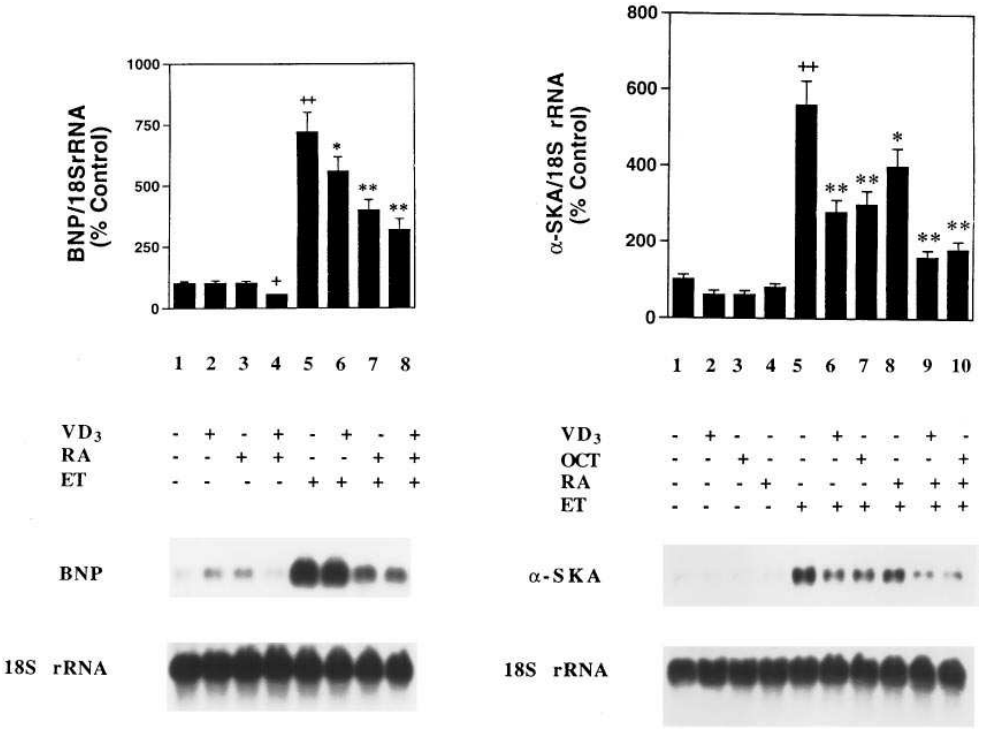

Figure 3. Effect of all-*trans* RA and/or vitamin D derivatives on ET-stimulated expression of BNP (*left*) and α-skeletal actin (*right*) genes in cultured ventricular myocytes. Cells were treated and RNA was prepared as described in Fig. 2. Where indicated OCT (10^{-8} M) was substituted for 1,25 $(OH)_2$ vitamin D_3. 15 μg RNA from each group was size fractionated, transferred to a nitrocellulose filter, and blot hybridized with [α-^{32}P]dCTP-labeled cDNAs for BNP and 18S ribosomal RNA or a [γ-^{32}P]ATP-labeled antisense oligonucleotide for α-skeletal actin. Histogram depicts average values obtained from densitometric scans of two to three experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ET group.

cytes before treating the cultures with 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA. To amplify the effects of the latter, in selected experiments we cotransfected expression plasmids encoding the human VDR, human RAR (RARα), or the human RXR (RXRα). As shown in Fig. 5 *A*, 1,25 $(OH)_2$ vitamin D_3 and all-*trans* RA effected a modest and dose-dependent reduction in hANP promoter activity which reached statistical significance only at the highest concentrations of the individual agonists (i.e., 30% inhibition at 10^{-8} M VD_3 and 33% inhibition at 10^{-6} M RA). When the two were used together, amplification of the inhibitory activity was seen, at least at lower agonist concentrations. For example, while neither 10^{-10} M VD_3 nor 10^{-9} M RA effected a significant reduction in promoter activity, the combination reduced activity by 32% implying at least additive and possibly synergistic interactions between these two classes of agonists. Similarly, higher concentrations of agonist (e.g., 10^{-8} M VD_3 + 10^{-6} M RA) displayed at least additivity in the response. Cotransfection of either VDR or RAR (Fig. 5 *B*) increased the response to ligand (i.e., 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA) only modestly (10–15% in most instances) and, in this particular study (see Fig. 5, *A* and *B*), was significant only at the highest agonist concentrations [VD_3 (10^{-8} M) + VDR vs. VD_3 alone, $P < 0.05$; RA (10^{-6} M) + RAR vs. RA alone, $P < 0.05$]. Interestingly, cotransfection of subsaturating concentrations of liganded VDR [i.e., VDR + VD_3 (10^{-10} M)] together with liganded RAR led to a clear amplification of the inhibitory activity over that seen with liganded RAR alone. This effect persisted to some degree even at higher concentrations of liganded VDR [i.e., VDR + VD_3 (10^{-8} M)] implying an important interaction between these two classes of receptors. While cotransfection of unliganded VDR and RXR, alone or in combination, resulted in only modest changes in reporter activity, liganded RXR (10^{-5} M all-*trans* RA) was more effective than either liganded VDR or liganded RAR in suppressing hANP promoter activity (Fig. 5 *C*) and this effect, once again, was amplified in the presence of liganded VDR.

OCT had an effect on hANP-CAT reporter activity (Fig. 5 *D*) which was roughly equivalent to that seen with equimolar concentrations of 1,25 $(OH)_2$ vitamin D_3 (compare with Fig. 5, *B* and *C*) supporting the findings seen with the α-skeletal actin transcript in Fig. 4 and suggesting that 1,25 $(OH)_2$ vitamin D_3 activity in this system is largely independent of its calcium mobilizing properties. 9-*cis* RA, which activates RXR at the dose used here (10^{-8} M), provided a level of inhibition which was roughly equivalent to that seen with the all-*trans* derivative and, once again, there was an additional modest increase in the inhibition when both ligand/receptor systems were activated in parallel.

When the cotransfection analysis was extended to a human BNP-CAT reporter system, very similar results were obtained (Fig. 6 *A*). In this case the liganded VDR was more effective in reducing reporter activity (vs. hANP-CAT reporter described above) and, once again, the combination of 1,25 $(OH)_2$ vitamin D_3 and RA resulted in an increase in inhibitory activity above and beyond that seen with either of the individual agonists alone. The α-skeletal actin–CAT reporter was suppressed by the liganded VDR while the liganded RXR was devoid of activity (Fig. 6 *B*); however, the combination of 1,25 $(OH)_2$ vitamin D_3 and RA once more provided a level of inhibition which was greater than that seen with either liganded receptor alone. These findings suggest that liganded VDR and RXR are capable of suppressing the expression of a number of hypertrophy-activatable genes and that this occurs, at least in part, through a reduction in transcriptional activity. The inhibition was specific to this subset of myocardial genes. When a reporter plasmid (DR3CAT) linking a conventional VDRE upstream from a TK promoter–driven CAT reporter (−109TK CAT) was cotransfected into ventricular myocytes together with liganded VDR and/or RXR, there was an increase rather

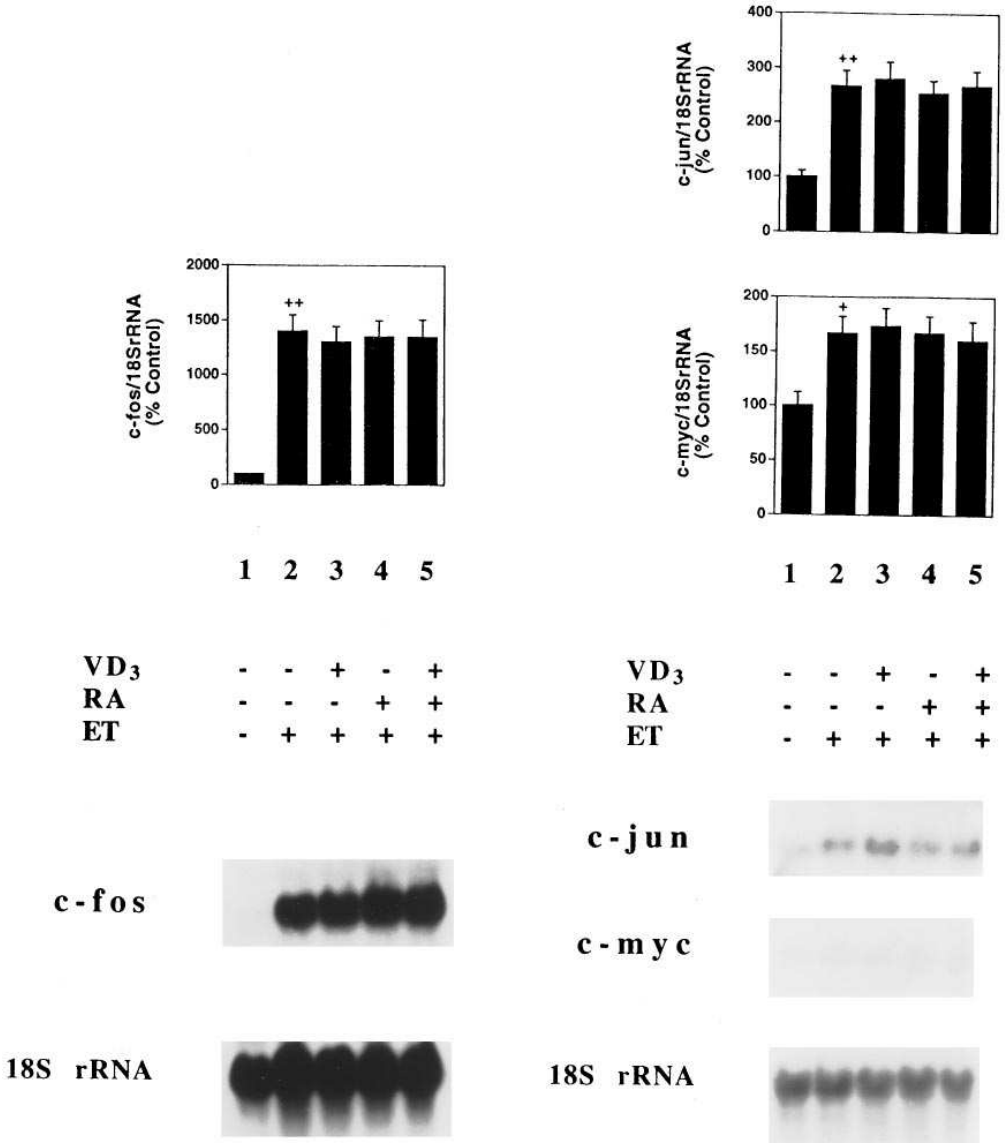

Figure 4. Effect of 1,25 $(OH)_2$ vitamin D_3 and/or all-*trans* RA on ET-stimulated expression of protooncogene products in cultured ventricular myocytes. Cells were treated and RNA was prepared as described in Fig. 2. 15 μg RNA from each group was size fractionated, transferred to a nitrocellulose filter, and blot hybridized with radiolabeled cDNAs for c-*fos*, c-*jun*, c-*myc*, or 18S ribosomal RNA. Histograms depict averages obtained from densitometric scans of two to three experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control.

than a reduction in reporter expression (Fig. 6 *C*). Similar experimental manipulation had no effect on the −109TK CAT background vector. Taken together these studies provide support for the selectivity of the inhibitory effect.

In addition to the specific effects on gene expression noted above, hypertrophy of the cardiac myocyte is accompanied by an increase in protein and RNA synthesis, an increase in cell size (14), and a reorganization of sarcomeric structure (16, 23). ET as a surrogate stimulus for hypertrophy in vitro has been shown to activate each of these responses in neonatal ventricular myocyte cultures (16). In an effort to extend the findings presented above, we examined the effects of 1,25 $(OH)_2$ vitamin D_3 and RA on [^{3}H]leucine incorporation, as an index of protein synthesis, in cultured ventricular myocytes. As shown in Fig. 7 *A*, ET treatment of these cultures resulted in a significant increase in [^{3}H]leucine incorporation. This was reversed in a dose-dependent fashion by increasing concentrations of 1,25 $(OH)_2$ vitamin D_3 after 48 h of treatment with the secosteroid. A more extensive time course (Fig. 7 *B*) revealed that the inhibition was apparent at 24 h and maximal at the 48 h time point. A point of potential concern here relates to possible 1,25 $(OH)_2$ vitamin D_3 effects on the growth and proliferation of nonmyocyte cells (e.g., cardiac fibroblasts which may harbor VDRs) which routinely contaminate our cultures at low levels. To address this concern we treated cultures enriched for the presence of cardiac fibroblasts (see Methods) according to the same protocol used for the myocytes above. Of interest, ET was effective in stimulating [^{3}H]leucine incorporation into protein in these cells (Fig. 7 *C*); however, 1,25 $(OH)_2$ vitamin D_3 was unable to reverse this stimulation, suggesting that the 1,25 $(OH)_2$ vitamin D_3–dependent inhibition is largely confined to the myocyte population in the heart.

Like 1,25 $(OH)_2$ vitamin D_3, RA had little effect on basal [^{3}H]leucine incorporation (Fig. 8); however, it fostered a modest, dose-dependent reduction in ET-stimulated protein synthesis. The magnitude of this reduction was on the same order (30%) as that seen with 1,25 $(OH)_2$ vitamin D_3. Once again, the combination of 1,25 $(OH)_2$ vitamin D_3 and RA displayed improved efficacy versus either agent alone.

Finally, we sought to obtain a morphological correlate of the biochemical data presented above. Previous studies using an antiserum directed against the sarcomeric myosin light

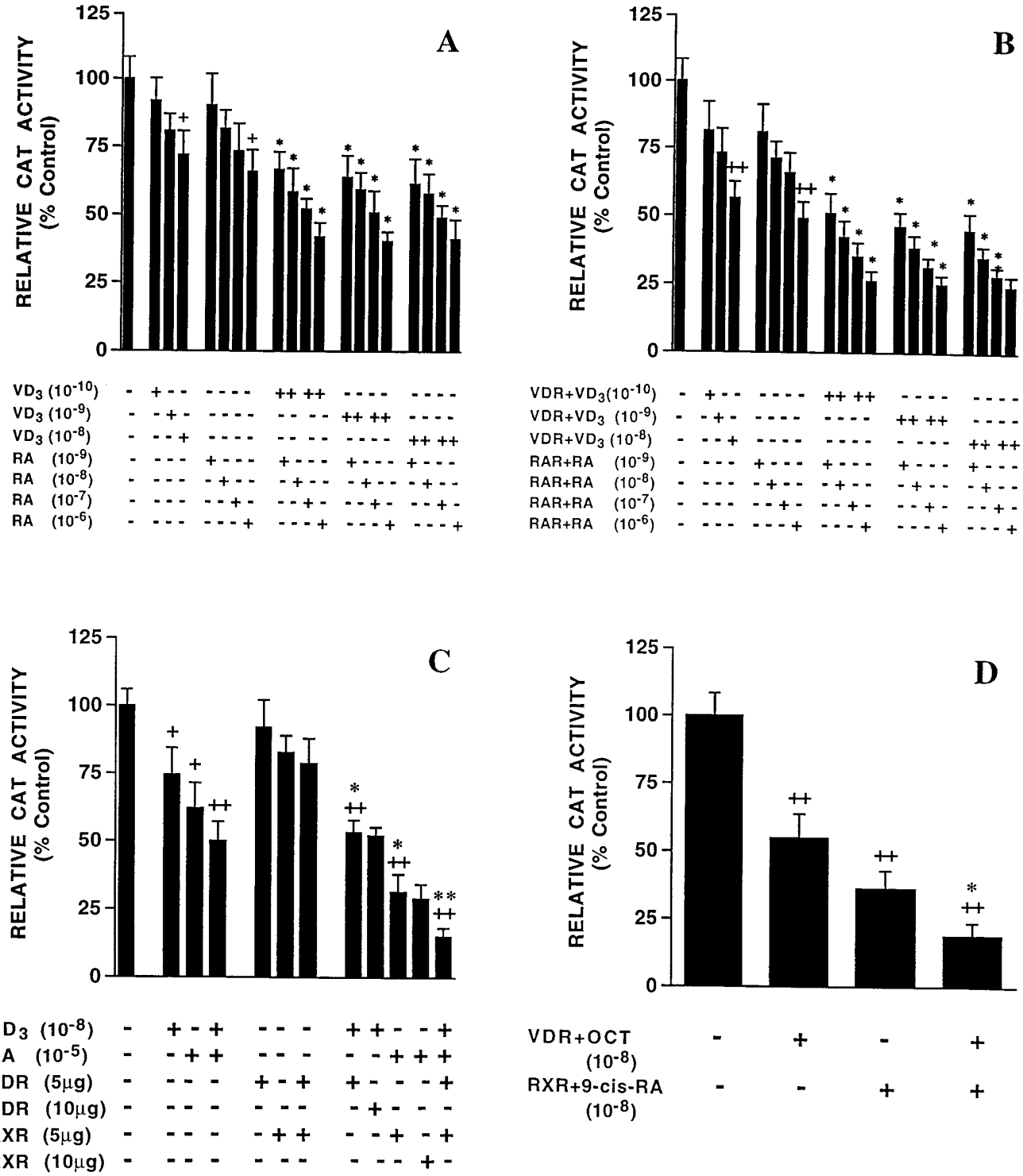

Figure 5. Effect of 1,25 $(OH)_2$ vitamin D_3, OCT, and/or RA on hANP gene promoter activity. (*A*) Freshly isolated ventricular myocytes were transfected with 20 μg −1150 hANP CAT. Different concentrations of 1,25 $(OH)_2$ vitamin D_3, all-*trans* RA, or combinations thereof were added to the culture medium 48 h before harvesting the cells for CAT assay. Data represent means±SD from three different experiments. $^{+}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. corresponding RA group. (*B*) Freshly isolated ventricular myocytes were transfected with 20 μg −1150 hANP CAT alone or cotransfected together with VDR (5 μg) and/or RAR (5 μg) expression vector. Where appropriate, exogenous ligand [1,25 $(OH)_2$ vitamin D_3 or all-*trans* RA], at the concentrations indicated, was added to the culture medium 48 h before harvesting the cells for CAT assay. Data represent means±SD from three different experiments. $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. corresponding RAR + RA group. (*C*) Freshly isolated ventricular myocytes were transfected with 20 μg −1150 hANP CAT alone or cotransfected together with VDR (5–10 μg) and/or RXR (5–10 μg) expression vector. Where appropriate, exogenous ligand [1,25 $(OH)_2$ vitamin D_3 or all-*trans* RA], at the concentrations indicated, was added to the culture medium 48 h before harvesting the cells for CAT assay. Data represent means±SD from three different experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding ligand alone group. (*D*) Effect of OCT and/or 9-*cis* RA on ANP promoter activ-

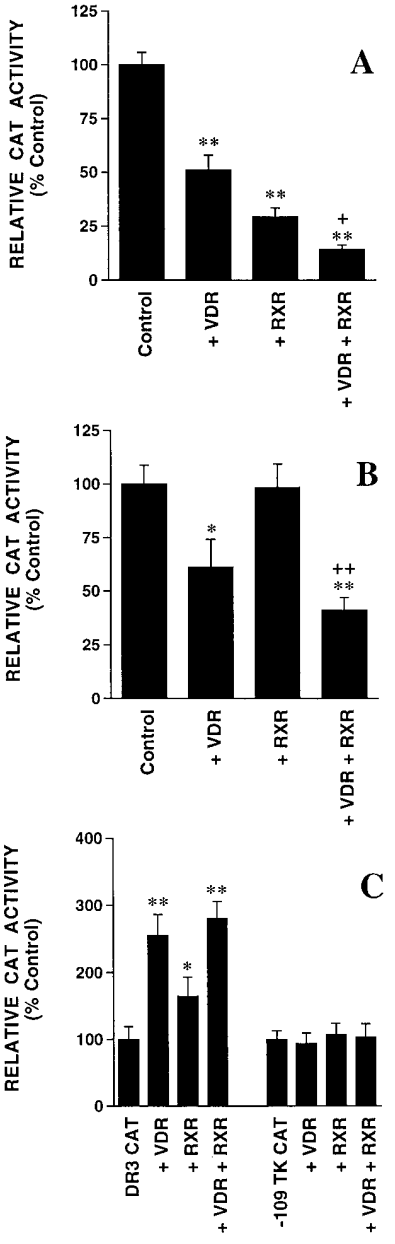

Figure 6. Effect of 1,25 $(OH)_2$ vitamin D_3 and/or RA on target gene promoter activity. Protocols identical to those used in Fig. 5 were carried out for −1173 hBNP CAT (*A*), −1400 α-skeletal actin CAT (*B*), DR_3CAT (*C*), or TK CAT (*C*). All values are expressed as a percentage of the CAT activity in the relevant control group. Data represent means±SD from three to six different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{+}P < 0.05$, $^{++}P < 0.01$ vs. RXR alone.

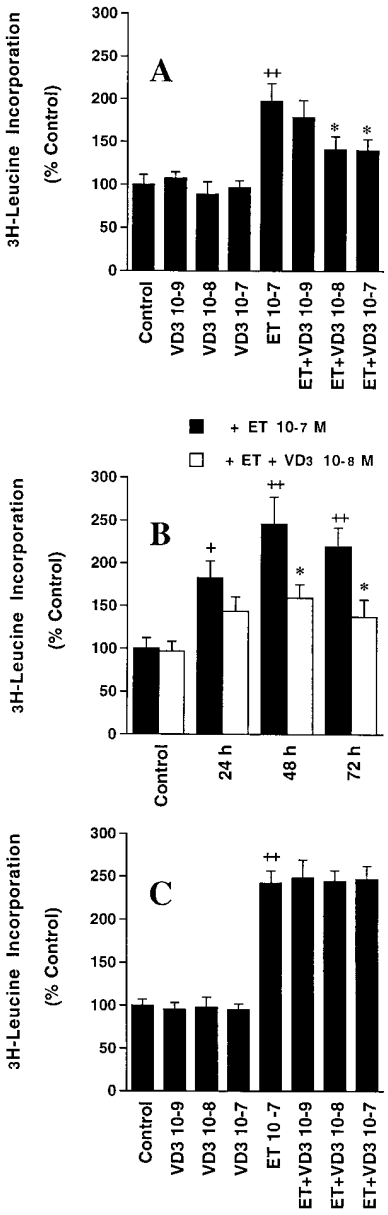

Figure 7. Effect of 1,25 $(OH)_2$ vitamin D_3 on ET-stimulated [^{3}H]leucine incorporation into cultured cardiac cells. (*A*) Ventricular myocytes were treated with increasing concentrations of 1,25 $(OH)_2$ vitamin D_3 for 48 h in serum-free medium. ET (10^{-7} M) was added to medium for the final 24 h of the incubation. Cells were then pulsed with [^{3}H]leucine (1 μCi/well) in leucine-free medium containing the same additives, as indicated, for the last 4 h. (*B*) Myocytes were treated with 1,25 $(OH)_2$ vitamin D_3 for different periods of time in serum-free medium and, as above, 10^{-7} M ET was included in the medium for the final 24 h. Pulse labeling with [^{3}H]leucine was carried out as described above. (*C*) Cultured cardiac fibroblasts were subjected to the same experimental protocol as described in *A*; cells were pulsed and [^{3}H]leucine incorporation was measured as described. All values are expressed as a percentage of the incorporation in control group. Data represent means± SD from six different experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. ET (10^{-7} M).

chain-2 protein demonstrated that exposure of neonatal ventriculocytes to a hypertrophic stimulus resulted in an increase in both the size and sarcomeric organization of the cells (23). To approach this question in our system, we stained cells with a fluorescein-labeled phalloidin tag both before and after treatment with ET and in the presence or absence of 1,25 $(OH)_2$ vitamin D_3. Phalloidin is a mushroom toxin which selectively decorates polymerized actin molecules in the cell (44). It has been used successfully as a probe of cytoskeletal organization in neonatal cardiac myocyte cultures (45). As shown in Fig. 9, treatment of the cultures with ET resulted in an increase in cell size and an increase in both the staining intensity and the organization of the actin fibers in the cytoplasm of these cells. While 1,25 $(OH)_2$ vitamin D_3 alone had little effect on the actin staining pattern in these cells, it caused a partial reversal of the changes promoted by ET. There was a significant decrease in cell size; however, the staining intensity was not attenuated significantly in the 1,25 $(OH)_2$ vitamin D_3–treated cells. Subsequent measurement of two-dimensional surface area of individual cells in each of the four groups substantiated the impression obtained from inspection of the photomicro-

ity. Freshly isolated ventricular myocytes were transfected with 20 μg −1150 hANP CAT alone or cotransfected together with VDR (5 μg) and/or RXR (5 μg) expression vector. In each instance where exogenous receptor was expressed, the appropriate ligands (10^{-8} M OCT for VDR and 10^{-8} M 9-*cis* RA for RXR) were added to the culture medium 48 h before harvesting the cells for measurement of CAT assay. All values are expressed as a percentage of the CAT activity in the control group. Data represent means±SD from four different experiments. $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. liganded RXR or VDR alone.

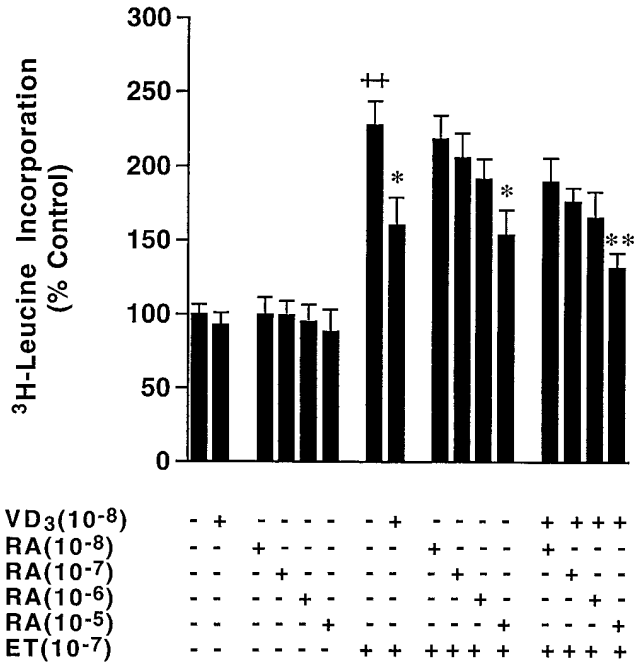

Figure 8. Effect of all-*trans* RA on ET-stimulated [^{3}H]leucine incorporation in cultured ventricular myocytes. Cells were treated with all-*trans* RA in the presence or absence of 1,25 $(OH)_2$ vitamin D_3 (10^{-8} M) for 48 h in serum-free medium. ET (10^{-7} M) was added to the medium for the final 24 h. Cells were pulsed with [^{3}H]leucine as described in Fig. 7. Data are expressed relative to control [^{3}H]leucine incorporation and represent means ± SD from six experiments. $^{++}P <$ 0.01 vs. control; $^*P < 0.05$, $^{**}P < 0.01$ vs. ET (10^{-7} M).

graph (Table I). This indicates that the 1,25 $(OH)_2$ vitamin D_3–dependent antagonism of ET's hypertrophy-promoting activity has important structural correlates in the intact cell.

Discussion

Reactivation of ANP secretion and ANP gene expression are two of the most reliable early markers of hypertrophy in ventricular myocytes (16–19). While a number of hemodynamic, mechanical, and biochemical stimuli have demonstrated the ability to activate ANP gene expression, as well as the downstream events in the cascade, until now there has been little information available regarding potential antagonists, particularly endogenous antagonists, of hypertrophy. This study suggests that retinoids and, to some extent, 1,25 $(OH)_2$ vitamin D_3 may play such a role. We have assessed hypertrophy through a number of independent measurements including changes in ANP secretion, changes in ANP, BNP, and α-skeletal actin mRNA levels and promoter activity, changes in [^{3}H]leucine incorporation into protein, and changes in cell size and cytoskeletal organization. In each case ET treatment provided a hypertrophy-like phenotype, and in each case 1,25 $(OH)_2$ vitamin D_3 and/or RA abrogated this response.

Retinoids are small lipophilic molecules thought to play an important part in growth and differentiation during development. They bind with high affinity to one of two nuclear receptor types called RAR and the RXR. Each of these, in turn, includes three different subtypes (α, β, γ). RXR has drawn particular attention of late by virtue of its ability to associate with and amplify the activity of a number of different receptors belonging to the thyroid hormone receptor family (2). Of relevance to the present studies, RXR gene knockouts have been shown to result in serious perturbations in cardiac morphogenesis in transgenic mice. Mice, homozygous for deletion of the RXR locus, die between embryonic days 13.5 and 16.5 with cardiac hypoplasia and presumed heart failure (9, 10). In some instances these alterations in phenotype can also be found in compound RAR knockouts (46). The developmental abnormalities are even more prominent in mice bearing deletions in both the RAR and RXR loci (10), implying a convergence of retinoid receptor signaling systems in establishing morphogenetic patterns in the developing cardiovascular system. The retinoid receptors recognize different ligands with varying levels of affinity. 10^{-8} M all-*trans* RA has been shown to be saturating for the RAR (41). RXR, on the other hand, recognizes 9-*cis* RA as its natural ligand and does not bind all-*trans* RA to a significant degree. At high concentrations (i.e., 10^{-5} M) all-*trans* RA activates RXR by virtue of low level chemical isomerization of the agonist (i.e., conversion of all-*trans* to 9-*cis* RA) (41). By virtue of the high concentrations of all-*trans* RA required to see the inhibitory effect and the greater efficacy of cotransfected RXR versus RAR in amplifying this inhibition, we believe that RXR is the more potent of these two receptors in mediating this phenomenon. However, it is clear that liganded RAR, like RXR, is capable of augmenting the response to liganded VDR and, of equal importance, this effect is apparent at RA concentrations which would be predicted to saturate RAR. Thus, several members of this extended nuclear receptor family appear capable of participating in this regulatory phenomenon. The level of inhibition may depend, in large part, on the nature of the individual receptor components present in the heterodimeric complexes which mediate this effect.

1,25 $(OH)_2$ Vitamin D_3 has been noted to possess both growth-promoting and growth-suppressant activity in different systems. The direction of the response appears to be a function both of the type of target cell under study and the characteristics of the culture medium. In the cardiac myocyte 1,25 $(OH)_2$ vitamin D_3 displays predominantly suppressive activity, at least under the conditions chosen for the present experiments. We have not carried out an exhaustive investigation of 1,25 $(OH)_2$ vitamin D_3 activity under different culture conditions and it is conceivable that the magnitude and/or direction of the effect may vary as such conditions are modified.

It is somewhat surprising that the effect of 1,25 $(OH)_2$ vitamin D_3 on ANP, as well as BNP, mRNA levels was less than that seen with RA since the two agonists were nearly equivalent in suppressing ANP secretion, hANP promoter activity, and [^{3}H]leucine incorporation. We have no explanation for this but it may suggest that these agents operate with variable efficacy at several loci in the hypertrophic cascade. Thus, while the phenotype of the cells is ultimately quite similar after treatment with RA vs. 1,25 $(OH)_2$ vitamin D_3, the pathways which they use to arrive at these phenotypes are to some degree unique.

Previous studies have suggested a potentially important role for 1,25 $(OH)_2$ vitamin D_3 in cardiovascular function. Several groups have identified VDRs in cardiac myocytes (3) and 1,25 $(OH)_2$ vitamin D_3 has been shown to have significant effects on $^{45}Ca^{2+}$ flux in these cells (5). Receptors have also been described in vascular smooth muscle cells, where 1,25 $(OH)_2$

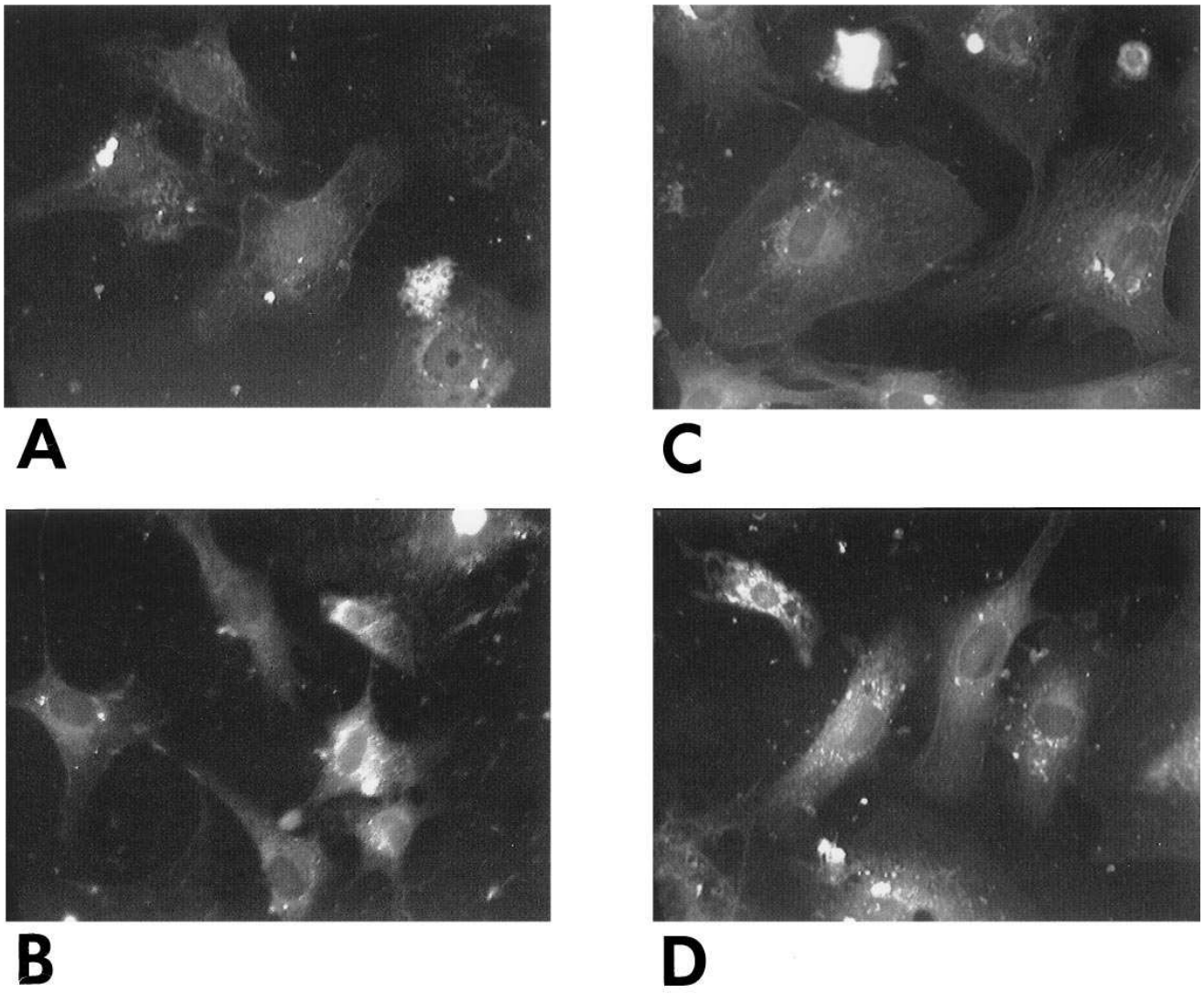

Figure 9. Effect of 1,25 $(OH)_2$ vitamin D_3 on morphology of cultured ventricular myocytes. Cells were treated with 1,25 $(OH)_2$ vitamin D_3 for 48 h in serum-free medium. ET (10^{-7} M) was added to the medium for the final 24 h. Cells were then fixed on the slide with 3.7% formaldehyde solution, stained with fluorescein-tagged phalloidin, and viewed by fluorescence microscopy. *A*, Control; *B*, 1,25 $(OH)_2$ vitamin D_3 (10^{-8} M); *C*, ET (10^{-7} M); *D*, 1,25 $(OH)_2$ vitamin D_3 plus ET.

vitamin D_3 plays a role in regulating protooncogene expression, cell growth, and mitogenesis (47, 48). The nature of the regulatory activity in the latter case has been quite variable with some studies reporting growth-promoting and others growth-suppressing activity. As discussed above, this likely reflects differences in the culture conditions used for the individual experiments. Mitsuhashi et al. (47) reported that while 1,25 $(OH)_2$ vitamin D_3 stimulated [^{3}H]thymidine incorporation in serum-starved vascular smooth muscle cells, it actually suppressed synthesis which had been preactivated with epidermal growth factor. The molecular underpinnings of this divergent behavior remain undefined. VDRs are also present in vascular endothelial cells and preliminary evidence (49) suggests that these cells may also possess 1-hydroxylase activity [i.e., the capacity to synthesize 1,25 $(OH)_2$ vitamin D_3]. If confirmed, the presence of such activity would raise the possibility of an important paracrine interaction between the endothelial lining of the vasculature and neighboring vascular smooth muscle or myocardial cells.

Table I. Summary of 1,25 $(OH)_2$ VD_3 Effects on Cell Size

	No. of cells	Relative cell size
Control	8	100±12
VD_3 (10^{-8} M)	12	98±8
ET (10^{-7} M)	10	270±30*
ET(10^{-7} M) + VD_3 (10^{-8} M)	11	120±14‡

Cell size was estimated as described in the text and expressed as a function of that determined in untreated (Control) cultures. Data are means±SEM. *$P < 0.01$ vs. Control; ‡$P < 0.01$ vs. ET (10^{-7} M).

Weishaar et al. (7) reported that 1,25 $(OH)_2$ vitamin D_3 deficiency in otherwise intact rats results in an increase in peripheral vascular resistance, cardiac contractility, and blood pressure. When these animals were provided with exogenous calcium to correct the hypocalcemia which accompanied the 1,25 $(OH)_2$ vitamin D_3 deficiency, the cardiac contractile ab-

normalities persisted, implying that at least one physiological consequence of 1,25 $(OH)_2$ vitamin D_3 activity in the heart is calcium independent. Our previous studies in atrial myocytes showed that calcium was not necessary either for the inhibition of irANP release or for suppression of hANP gene promoter activity (26). This is corroborated in the present study by the fact that OCT, a nonhypercalcemic analogue of 1,25 $(OH)_2$ vitamin D_3, retains the ability to suppress both α-skeletal actin mRNA levels and transcriptional activity of the hANP promoter. Taken together these data suggest that at least a portion of 1,25 $(OH)_2$ vitamin D_3 activity in the cardiac myocyte is independent of its ability to effect changes in calcium transport.

The mechanism underlying the suppression of genes in the fetal gene program remains undefined. As noted previously (26, 27), we have been unable to identify typical VDRE-like structures in the proximal 5′ FS of the hANP gene, a region which covers sequence which is functionally sensitive to inhibition by 1,25 $(OH)_2$ vitamin D_3. Similarly, we have been unable to demonstrate high-affinity association of radiolabeled VDR with this region of the promoter (data not shown). This may imply that the VDR effect is indirect, operating through an intermediate which, itself, displays more conventional VDRE-dependent regulation. Alternatively, it may suggest that the VDR (perhaps in concert with RXR) exerts its effects through interaction with key positive regulatory proteins which are required for expression of each of the genes in the repertoire.

Very recently Zhou et al. (50) published a study which bears directly on those presented here. They found that RA suppressed phenylephrine-induced increments in ventricular myocyte size, ANP gene expression, and rat ANP promoter activity. They also found that the RA-mediated reduction in cell size was not accompanied by suppression of phenylephrine-mediated increases in myofibrillar organization. Our studies with 1,25 $(OH)_2$ vitamin D_3 demonstrated a similar reduction in cell size without alteration in ET-mediated cytoskeletal organization. Of interest, while the RA-dependent inhibition extended to both phenylephrine- and ET-activated hypertrophy, it was not effective in reversing serum-mediated hypertrophy, implying that these various stimuli may use different mechanistic pathways to evoke a similar phenotypic response in the cardiac myocyte. In contrast to our findings, Zhou et al. (50) found that RAR was the more important of the two retinoid receptors in suppressing ANP promoter activity. This conclusion was based on greater efficiency of an RAR-selective (TTTPB), versus RXR-selective (LG64), ligand in suppressing phenylephrine-stimulated ANP promoter activity. As noted above, based on the dose sensitivity of the response to all-*trans* RA and greater efficacy of liganded RXR versus RAR in suppressing hANP transcriptional activity, we believe that RXR plays a major role in mediating RA-dependent suppression of the hypertrophic phenotype. However, it is clear that in certain experimental paradigms (e.g., in the presence of liganded VDR) RAR can participate more actively in the inhibition (see Fig. 5 *C*), perhaps reflecting its ability to form heterodimeric complexes with these heterologous receptors (51). It is conceivable that at least part of the discrepancy between the findings of Zhou et al. (50) and our own results from differential availability of as yet undefined heterodimeric partners in the two culture systems. Finally, Zhou et al. (50) found no effect of vitamin D_3 in reversing the hypertrophic phenotype. Our studies clearly show that this ligand does exert antihypertrophic activity which is qualitatively similar to that seen with all-*trans* RA, although quantitatively, at least with regard to selected markers of hypertrophy (e.g., ANP transcript levels), RA appears to be the more potent of the two agonists.

In summary, our data indicate that many parameters associated with the activation of hypertrophy in a cultured neonatal rat cardiac myocyte model are suppressed by 1,25 $(OH)_2$ vitamin D_3 and/or RA. This raises the intriguing possibility that one (or both) of these agents, or a related ligand-receptor system, may participate in the regulatory circuitry which controls growth in the myocardium. Such involvement may suggest novel pharmacological approaches for the management of hypertrophy and remodeling in the heart.

Acknowledgments

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