Comparison of 6-s-*cis*- and 6-s-*trans*-Locked Analogs of 1α,25-Dihydroxyvitamin D_3 Indicates That the 6-s-*cis* Conformation Is Preferred for Rapid Nongenomic Biological Responses and That Neither 6-s-*cis*- nor 6-s-*trans*-Locked Analogs Are Preferred for Genomic Biological Responses

Anthony W. Norman, William H. Okamura, Marion W. Hammond, June E. Bishop, Murray C. Dormanen,* Roger Bouillon, Hugo van Baelen, Amy L. Ridall, Elizabeth Daane, Ramzi Khoury, and Mary C. Farach-Carson

Division of Biomedical Sciences (A.W.N.)
Departments of Biochemistry (A.W.N., J.E.B., M.C.D., M.C.F-C.) and Chemistry (W.H.O., M.W.H.)
University of California
Riverside, California 92521

Laboratorium voor Experimentele Geneeskunde en Endocrinologie (R.B., H.v.B.)
Katholieke Universiteit Leuven
Leuven B-3000, Belgium

Department of Basic Sciences (A.L.R., E.D., R.K., M.C.F-C.)
University of Texas Dental Branch
Houston, Texas 77030

The hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] generates biological responses via both genomic and rapid, nongenomic mechanisms. The genomic responses utilize signal transduction pathways linked to a nuclear receptor (VDR_{nuc}) for 1α,25$(OH)_2D_3$, while the rapid responses are believed to utilize other signal transduction pathways that may be linked to a putative membrane receptor for 1α,25$(OH)_2D_3$. The natural seco steroid is capable of facile rotation about its 6,7 single carbon bond, which permits generation of a continuum of potential ligand shapes extending from the 6-s-*cis* (steroid like) to the 6-s-*trans* (extended). To identify the shape of conformer(s) that can serve as agonists for the genomic and rapid biological responses, we measured multiple known agonist activities of two families of chemically synthesized analogs that were either locked in the 6-s-*cis* (6C) or 6-s-*trans* (6T) conformation. We found that 6T locked analogs were inactive or significantly less active than 1α,25$(OH)_2D_3$ in both rapid responses (transcaltachia in perfused chick intestine, $^{45}Ca^{2+}$ influx in ROS 17/2.8 cells) and genomic (osteocalcin induction in MG-63 cells, differentiation of HL-60 cells, growth arrest of MCF-7 cells, promoter transfection in COS-7 cells) assays. In genomic assays, 6C locked analogs bound poorly to the VDR_{nuc} and were significantly less effective than 1α,25$(OH)_2D_3$ in the same series of assays designed to measure genomic responses. In contrast, the 6C locked analogs were potent agonists of both rapid response pathways and had activities equivalent to the conformationally flexibile 1α,25$(OH)_2D_3$; this represents the first demonstration that 6-s-*cis* locked analogs can function as agonists for vitamin D responses. (Molecular Endocrinology 11: 1518–1531, 1997)

0888-8809/97/$3.00/0
Molecular Endocrinology

INTRODUCTION

The seco steroid[1] vitamin D_3 is important for many biological processes in higher animals, including maintenance of calcium homeostasis, selected cell differentiation, and immunomodulation (1, 2). However, the parent vitamin D_3 is biologically inert, and it is only as a consequence of its metabolism to $1\alpha,25(OH)_2D_3$[2] and other metabolites that its biological activity is achieved (2).

It is well established that $1\alpha,25(OH)_2D_3$ can stimulate biological responses via signal transduction pathways that utilize nuclear receptors for $1\alpha,25(OH)_2D_3$ (VDR_{nuc}) to regulate gene transcription (3–5). Indeed the VDR_{nuc} for $1\alpha,25(OH)_2D_3$ belongs to a superfamily of receptors for the steroid hormones, retinoic acid, and T_4 (5, 6).

Also there is considerable evidence that $1\alpha,25(OH)_2D_3$ can utilize different signal transduction pathways to generate rapid, nongenomic biological responses. Rapid actions of $1\alpha,25(OH)_2D_3$ have been reported in a variety of systems including: 1) stimulation of intestinal Ca^{2+} transport in the perfused chick intestine (termed transcaltachia) (7–9), which involves the associated rapid opening of Ca^{2+} channels (10, 11); 2) a prolonged opening of both voltage-gated Ca^{2+} channels (12, 13) and chloride channels (14) in rat osteosarcoma cells; 3) rapid effects on $^{45}Ca^{2+}$uptake in ROS 17/2.8 cells (15), which is independent of the VDR_{nuc} effects on gene transcription of osteopontin (OPN) and osteocalcin (16); 4) rapid changes in intracellular Ca^{2+} concentrations in a pancreatic β-cell line (17); 5) rapid effects on phospholipid metabolism in the intestine (18), liver (19), parathyroid cells (20), and kidney (21); 6) rapid changes in membrane fluidity and protein kinase C (PKC) activity in chondrocytes (22); 7) rapid effects on the cellular redistribution of PKC (23–27); and 8) the direct activation of PKC in phospholipid bilayers (28).

The A ring, triene, and side chain of vitamin D_3 and all its metabolites are, in comparison to other steroid hormones (29–31), unusually conformationally mobile (32, 33). It is pertinent in a structure-function context to discern whether conformers of $1\alpha,25(OH)_2D_3$ differ in their ability to mediate biological responses, *i.e*. differ in their ability to interact with the VDR_{nuc} and the signal transduction process responsible for rapid responses. In this study, we focus on the conjugated triene system characteristic of vitamin D. Vitamin D seco steroids can undergo rotation about the 6,7 carbon-carbon single bond, which permits generation of a continuum of potential ligand shapes extending from the 6-s-cis (steroid-like conformation) to the the 6-s-trans (extended steroid conformation); see Fig. 1. We have previously presented a detailed study of the biological properties of a 6-s-*cis* locked analog, 1,25-$(OH)_2$-d_5-pre-D_3 (34) and reported that two rapid response/nongenomic systems respond as effectively to 1,25-$(OH)_2$-d_5-pre-D_3 as 1,25-$(OH)_2D_3$, while all tested genomic systems discriminated markedly against the 6-s-*cis* locked 1,25-$(OH)_2$-d_5-pre-D_3 species. This suggested that the ligand-binding domain of the VDR_{nuc} for 1,25-$(OH)_2D_3$ may be fundamentally different from the ligand-binding domain of the transducer, which is associated with rapid responses to $1\alpha,25(OH)_2D_3$.

In this communication we report the results of a detailed second level analysis of the biological properties of five new 6-s-*cis* analogs *vs.* two new 6-s-trans analogs of $1\alpha,25(OH)_2D_3$ to stimulate genomic and/or rapid biological responses. The results are consistent with the model that the genomic and nongenomic/rapid responses have distinct preferences with regard to the conformation of their agonist ligand. A preliminary communication has appeared (35).

RESULTS

This article presents a comparison of the biological profile, in relation to $1\alpha,25(OH)_2D_3$, of two classes of analogs; those that are 6-s-*cis* locked and those that are 6-s-*trans* locked. The structures of the seven analogs used in this report are given in Fig. 1. All seven analogs are newly synthesized, and this is the first comprehensive report of their biological properties; a preliminary report did appear concerning analogs **JM** and **JN** (35).

The four 6-s-*cis* locked analogs (**JM, JN**, **JO**, **JP**), are not seco steroids; thus they are permanently

[1] Secosteroids are by definition compounds in which one of the cyclopentanoperhydrophenanthrene rings of the steroid ring structure is broken. In the case of vitamin D_3 the 9–10 carbon-carbon bond of the B ring is broken generating a seco-B steroid. See Fig. 1 for examples. The official IUPAC name for vitamin D_3 is 9,10-secocholesta-5,7,10(19)-trien-3β-ol.

[2] The abbreviations used are 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3(analog **C**); 1,25-$(OH)_2$-pre-D_3, 1α,25-dihydroxy-previtamin-D_3 (analog **BC**); D_3, vitamin D_3; pre-D_3, previtamin-D_3; $1\alpha,25(OH)_2$-d_5-pre-D_3 1α,25-dihydroxy-9,14,19,19,19-pentadeuterio-previtamin-D_3 (analog **HF**); 1,25-$(OH)_2$-d_5-D_3, 1α,25-dihydroxy-9,14,19,19,19-pentadeuterio-vitamin-D_3, (analog **HG**); 1,25-trans-T, 1α,25-dihydroxytachysterol$_3$ (analog **JB**); 1,25-cis-isoT, $1\alpha,25(OH)_2$-cis-isotachysterol (analog **JC**); 1,25-trans-iso-T, 1α,25-dihydroxy-trans-isotachysterol$_3$ (analog **JD**); $1\alpha,25(OH)_2$-7-DHC, $1\alpha,25(OH)_2$-7-dehydrocholesterol (analog **JM**); 1α,25 $(OH)_2$-L, $1\alpha,25(OH)_2$-lumisterol$_3$ (analog **JN**); $1\alpha,25(OH)_2$-P $1\alpha,25(OH)_2$-pyrocalciferol$_3$ (analog **JO**); $1\alpha,25(OH)_2$-IP, $1\alpha,25(OH)_2$-isopyrocalciferol$_3$ (analog **JP**); DBP, vitamin D-binding protein from human (h), rat (r), or chick (c) serum; ICA, intestinal Ca^{2+} absorption; BCM, bone Ca^{2+} mobilization; transcaltachia, the rapid hormonal stimulation of intestinal Ca^{2+} absorption; VDR_{nuc}, nuclear receptor for $1\alpha,25(OH)_2D_3$; HRMS, high resolution mass spectrometry; ROS 17/2.8 cells, an osteogenic sarcoma cell line from rat; MCF-7 cell, a human breast adenocarcinoma cell line; COS-7 cells, a SV40 transformed African green monkey kidney cell line; HL-60 cells, a human promyelocytic cell line; MG-63 cells, a human osteosarcoma cell line; GBSS, Grey's balanced salt solution (see *Materials and Methods* section for composition); NBT, 4-nitro blue tetrazolium; OPN, osteopontin; rRNA, ribosomal RNA; VDRE, vitamin D response element; PKC, protein kinase C.

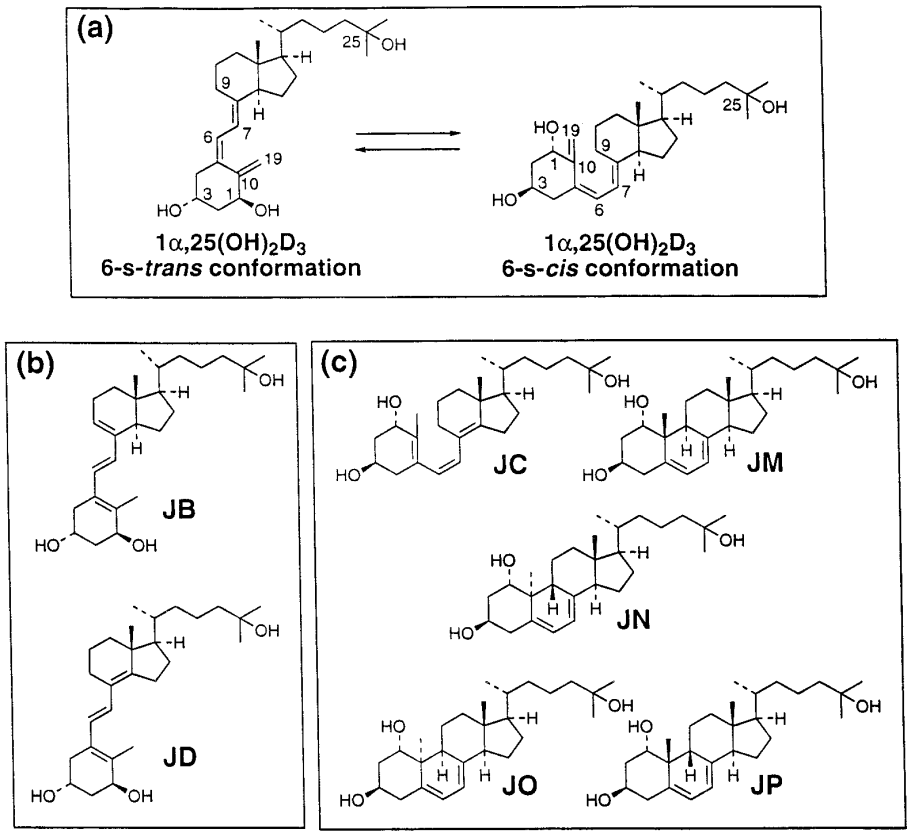

Fig. 1. Structures of $1\alpha,25(OH)_2D_3$ and Its Various Analogs That Are Evaluated in This Communication

Panel A, $1\alpha,25(OH)_2D_3$ is a conformationally mobile molecule with respect to the orientation of the A ring in relation to the C/D ring structure. The seco-B ring can assume in the limit one of two conformations as a consequence of a 360° rotation about the single bond between carbon 6 and carbon 7; in the 6-s-*cis* conformation the A ring is related to the C/D rings as in the conventional steroid orientation, referred to here as the "steroid-like conformation," and in the 6-s-*trans* presentation, the A ring is present in an "extended conformation". Panel B, Analogs **JB** and **JD** are seco steroids that possess a 6,7 double bond [in contrast to $1\alpha,25(OH)_2D_3$]. Depending upon the organization around the 6–7 double bond, the analogs may be locked in either the 6-s-*trans* conformation (**JB**, **JD**) or the 6-s-*cis* conformation. However **JB** and **JD** do display conformational mobility around their two 5,6 and 7,8 single carbon bonds, which permit them to generate a population of conformations not available to $1\alpha,25(OH)_2D_3$. Panel C, Analogs **JC**, **JM, JN**, **JO**, and **JP** are all 6-s-*cis* locked analogs. Because analogs **JM, JN**, **JO**, and **JP** are not seco steroids but provitamin D analogs, there is no possibility of rotation around the 6,7 single carbon bond; accordingly, they are all locked permanently in the 6-s-*cis* or "steroid-like conformation." In contrast, analog **JC** is a seco steroid because its 9,10 carbon bond is broken; however, because **JC** has a 6,7 double bond it is locked in a 6-s-*cis* conformation.

locked in the 6-s-*cis* conformation. They are also diastereomers with respect to the orientation of the hydrogen on C-9 and the methyl group on C-10. In **JM** and **JN**, the hydrogen atom at carbon 9 and the angular methyl group at carbon 10 are oriented on opposite faces (anti) of the pseudo plane defined by the steroid ABCD nucleus. More specifically, JM possesses the $9\alpha,10\beta$ (natural) configuration characteristic of steroids such as cholesterol; **JN** by contrast is doubly epimeric (9β, 10α) at these positions. In **JO** and **JP**, the corresponding hydrogen and methyl have a syn (on the same face) relationship to one another (9α, 10α, and $9\beta10\beta$, respectively) with respect to the pseudo plane of the steroid.

Analogs **JB**, **JC**, and **JD** are all seco steroids that possess a 6,7 double bond, in contrast to $1\alpha,25(OH)_2D_3$, and thus are locked in either the 6-s-*trans* (**JB, JD**) or the 6-s-*cis* (**JC**) conformation. Consequently, although **JB** and **JD** are not able to achieve interconversion to the 6-s-*cis* conformation like $1\alpha,25(OH)_2D_3$, they are able to display conformational mobility around their two 5,6 and 7,8 single-carbon bonds, which permits them to generate a population of conformations not available to $1\alpha,25(OH)_2D_3$. Similarly, **JC** can not achieve interconversion to the 6-s-*trans* conformation, but is also able to rotate around its 5,6 and 7,8 single carbon bonds so as to generate a different population of conformations not available to $1\alpha,25(OH)_2D_3$.

Biological Profile of New Analogs in Classic Vitamin D Assays

Table 1 summarizes the biological profile of the seven new analogs of $1\alpha,25(OH)_2D_3$ in four assays

Table 1. Relative Effects of $1\alpha,25(OH)_2D_3$ and Its B-Ring Analogs Upon Components of the Vitamin D Endocrine System

Compound Name	Analog Code	RCI DBP[a] (%)	RCI Intestine VDR^a_{nuc} (%)	Bioassay ICA[b] (%)	Bioassay BCM[b] (%)
$1\alpha,25(OH)_2D_3$	C	100	100	100	100
$1\alpha,25(OH)$-Tachysterol$_3$	JB	6.6 ± 1.1	0.01 ± 0.1	<0.3	<0.2
$1\alpha,25(OH)_2$-cis-Isotachysterol$_3$	JC	1.7 ± 0.8	0.41 ± 0.03	<0.5	<0.5
$1\alpha,25$-$(OH)_2$-trans-Isotachysterol$_3$	JD	−1.3 ± 3.9	1.0 ± 0.3	0.3	<0.2
$1\alpha,25(OH)_2$-7-Dehydrocholesterol	JM	−0.3 ± 0.1	0.12 ± 0.05	0.4 ± 0.1	0.4 ± 0
$1\alpha,25(OH)_2$-Lumisterol$_3$	JN	−0.7 ± 0.1	1.8 ± 0.5	5.0 ± 2.5	5.1 ± 2.2
$1\alpha,25(OH)_2$-Pyrocalciferol$_3$	JO	2.0 ± 0.7	0.23 ± 0.03	<0.4	<0.4
$1\alpha,25(OH)_2$-Isopyrocalciferol$_3$	JP	−5.4 ± 1.0	0.28 ± 0.06	0.5	0.5

[a] The results are expressed as the percentage activity for the relative competitive index for DBP and the VDR_{nuc} present in the chick intestine in comparison with $1\alpha,25(OH)_2D_3$ (RCI = 100%). The results are the mean ± SEM for three separate experiments conducted as described under *Materials and Methods*.

[b] The results presented for the bioassay of ICA and BCM are derived from *in vivo* dose-response studies carried out as described under *Materials and Methods* and Ref. 52. The results are expressed in terms of the dose of analog required to achieve an ICA or BCM response equivalent to that achieved by a 100 pmol dose of $1\alpha,25(OH)_2D_3$, calculated as a percentage, *i.e.* [$1\alpha,25(OH)_2D_3$ dose]/[analog dose] × 100. The results are the mean of two experiments or the mean ± SEM of three separate experiments.

that document the relative ability of the various analogs to bind *in vitro* to the chick intestinal $1\alpha,25(OH)_2D_3$ nuclear receptor (VDR_{nuc}) and the plasma transport protein, vitamin D binding protein (DBP), and to stimulate, under *in vivo* conditions in a vitamin D-deficient chick, the classic vitamin D responses of intestinal $^{45}Ca^{2+}$ absorption (ICA) and bone Ca^{2+} mobilization (BCM).

The $1\alpha,25(OH)_2D_3$ VDR_{nuc} is the mediator of genomic responses to $1\alpha,25(OH)_2D_3$ *in vivo* while the DBP is the principal protein that transports the hydrophobic vitamin D metabolites through the plasma compartment to target tissues. Both the VDR_{nuc} and the DBP are known to have ligand-binding domains that have different specificities for vitamin D analogs (2, 36). $1\alpha,25(OH)_2D_3$ is the reference compound and its relative competitive index (RCI) is, by definition, 100% for both the VDR_{nuc} and DBP assays (37).

None of the six analogs were able to compete effectively with [^{3}H]1,25-$(OH)_2D_3$ in the steroid competition assay for binding to the VDR_{nuc}; all RCI values were less than 2%, with four RCI values less than 0.5%. These results dramatically emphasize that analogs that are not seco steroids, as is the case for the four $1\alpha,25(OH)_2D_3$ 6-s-*cis* locked provitamins, are not effective ligands for the VDR_{nuc}. In addition, analog **JB**, which is a seco steroid, but which is a 6-s-*cis* locked analog by virtue of a double bond between carbons 6–7 and has a RCI of only 0.01%, also emphasizes that the 6-s-*trans* conformation is not preferred by the VDR_{nuc}. A similar conclusion can be proposed for the 6-s-*trans* locked analog **JD**, which has a RCI of only 1.0%. Apparently the VDR_{nuc} ligand-binding domain can not accommodate a 6-s-*trans* locked analog.

The RCI results for the DBP (Table 1) also emphasize that this protein's ligand-binding domain does not favor ligands with either a 6-s-*trans* or 6-s-*cis* conformation. With the exception of analog **JB**, for the other five analogs studied, all RCI values were $< \sim 2\%$, with four analogs (**JD**, **JM**, **JN**, **JP**) consistently reporting negative RCI values, suggesting a possible allosteric effect upon the binding of the [^{3}H]1,25-$(OH)_2D_3$ employed in the steroid competition assay.

Stimulation of ICA and BCM Activity Responses

The relative ability of the four 6-s-*cis* locked diastereomers to generate within 14 h the traditional vitamin D biological responses of ICA and BCM in the vitamin D-deficient chick, *in vivo*, was determined. It has been previously shown that both the ICA and BCM responses are largely genomic responses; both responses can be inhibited by administration of actinomycin D, an inhibitor of DNA-directed RNA synthesis (38). A portion of Table 1 summarizes the ICA and BCM results for the seven new analogs. The most potent stimulator of ICA and BCM, as expected, was the reference compound $1\alpha,25(OH)_2D_3$; the activity produced by 100 pmol of $1\alpha,25(OH)_2D_3$ was set to 100% for both ICA and BCM. The dose of the comparison analogs required to achieve a biological response of either ICA or BCM equivalent to the 100 pmol dose of $1\alpha,25(OH)_2D_3$ was then calculated and converted to a percentage. None of the analogs were potent agonists for either assay. The only analog that had a barely detectable agonist activity was analog **JN**, which had ≈5% of the activity of $1\alpha,25(OH)_2D_3$ for both ICA and BCM.

Rapid (Nongenomic) Actions

Figures 2 and 3 and Table 2 present an evaluation of the relative ability of the 6-s-*cis* and 6-s-*trans* locked analogs, in comparison to the conformationally flexible $1\alpha,25(OH)_2D_3$, to stimulate rapid nongenomic responses. Figure 2 focuses on the biological response of transcaltachia. Vascular perfusion at 650 pM with each of the four 6-s-*cis* locked analogs (Fig. 2A) resulted in a prompt stimulation within 2–5 min of

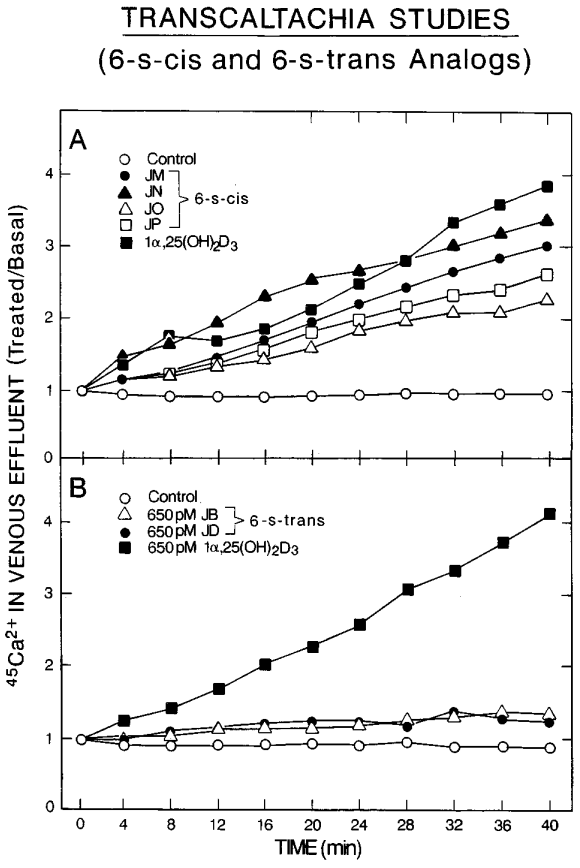

Fig. 2. Effect of Conformationally Locked Analogs of $1\alpha,25(OH)_2D_3$ on the Appearance of $^{45}Ca^{2+}$ in the Venous Effluent of Perfused Duodena from Vitamin D-Replete Chicks (Transcaltachia)

A, Evaluation of 6-s-*cis* locked $1\alpha,25(OH)_2$-provitamins D [**JM**, **JN**, **JO**, **JP**] and $1\alpha,25(OH)_2D_3$. B, Evaluation of 6-s-*trans* locked analogs [**JB**, **JD]** and $1\alpha,25(OH)_2D_3$. Each duodenum, filled with $^{45}Ca^{2+}$ (5 μCi/ml) in GBSS, was vascularly perfused (25 C) for the first 20 min with control medium (GBSS containing 0.125% BSA and 0.05 μl of ethanol/ml) and then at time zero with 650 pM of $1\alpha,25(OH)_2D_3$ or analog. See *Materials and Methods* for further details. In dose-response studies of the analogs carried out over 162, 325, 650, 1300, and 6500 pM, the maximum agonist concentration for all analogs was found to lie between 650-1300 pM (data not presented and Ref. 35). Values are the mean for n = 4 in each group. The definition of the symbols is provided in the figure panels. A summary is provided in Table 2.

transcaltachia and with a 1.8- to 4-fold increase in $^{45}Ca^{2+}$ over control levels at 40 min. Dose-response studies (data not shown) established that the maximum effective concentration for each analog was ~650 pM. Previous studies with $1\alpha,25(OH)_2D_3$ have established that the dose-response profile for transcaltachia is biphasic; thus, after the dose that generates a maximum response has been attained, higher concentrations of agonist result in lower responses (7, 9). Analog **JN** was found to be equipotent with $1\alpha,25(OH)_2D_3$, while the other three related diastereomers generated maximum transcaltachic responses that were diminished in relation to that of $1\alpha,25(OH)_2D_3$.

Figure 2B presents an evaluation of the 6-s-*trans* locked analogs **JB** and **JD** to stimulate transcaltachia. Neither **JB** nor **JD** displayed any agonist activity when perfused at a 650-6500 pM range. Collectively, the results presented in Fig. 2 suggest that the signal transducing element for transcaltachia does not recognize 6-s-*trans* locked agonists but can provide a full response, in comparison to $1\alpha,25(OH)_2D_3$, for selected 6-s-*cis* locked analogs, particularly the conformations presented by **JM** and **JN**.

Figure 3 presents an evaluation of the ability of the 6-s-*cis* analogs, in comparison to $1\alpha,25(OH)_2D_3$, to stimulate $^{45}Ca^{2+}$ uptake into ROS 17/2.8 cells within 1 min. As originally described by Caffrey and Farach-Carson (13), this response occurs as a consequence of the ability of $1\alpha,25(OH)_2D_3$ or its analogs to prolong the open time of dihydropyridine-sensitive Ca^{2+} channels via a rapid/nongenomic mechanism (see also Refs. 34 and 39). As shown in Fig. 3, analogs **JM** and **JN** stimulated $^{45}Ca^{2+}$ uptake in the concentration range of 0.01–100 nM. Analog **JN** consistently (over five experiments) displayed a biphasic ability to stimulate $^{45}Ca^{2+}$ influx, while analogs **JM**, **JO**, and **JP** (detailed data not shown) displayed only one concentration for maximum

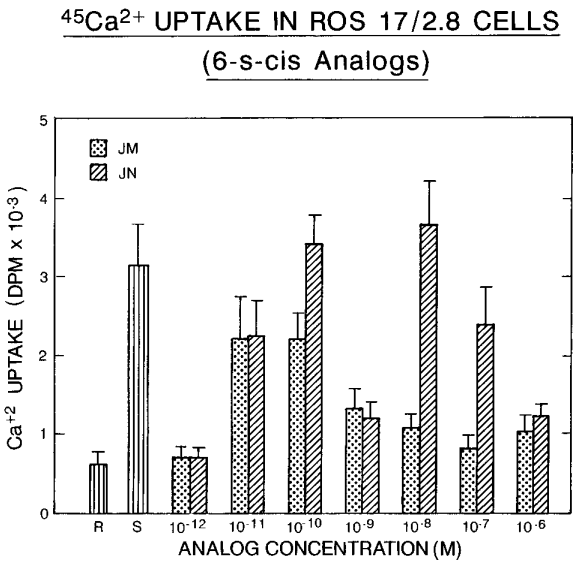

Fig. 3. Dose-Response Curves for 6-s-*cis* Analogs (**JM**, **JN**) in Assays of Transmembrane $^{45}Ca^{2+}$Influx in ROS 17/2.8 Cells

The ability of the analogs to stimulate influx of $^{45}Ca^{2+}$ during 1 min assays was measured as described in *Materials and Methods*. Doses were tested from 10^{-12} to 10^{-6} M. Error bars are ± SD for all data points at all concentrations over three to five experiments for the analogs. Results of identical uptake experiments in which cultures were treated either with resting buffer (R), depolarizing (high K^+) stimulating buffer (S) are also shown. The symbols are defined in the figure panels. The summary results for **JM**, **JN** as well as analogs **JB**, **JC**, **JD**, **JO**, **JP**, and $1\alpha,25(OH)_2D_3$ are presented in Table 2.

stimulation. The basis for the biphasic response of **JN** is not yet known.

Table 2 presents a summary of the relative activity of the seven new analogs in the two rapid response assays (transcaltachia and $^{45}Ca^{2+}$ uptake into ROS 17/2.8 cells). With regard to transcaltachia, the relative order of agonist effectiveness of the four 6-s-*cis* diastereomers is **JN** >**JM** >**JO** >**JP**; neither of the two 6-s-*trans* analogs (**JB** or **JD**) displayed significant transcaltachia activity. For the $^{45}Ca^{2+}$uptake, both the concentration of analog that achieved a maximum stimulation of $^{45}Ca^{2+}$uptake and the relative calcium index (RCX) are reported for all analogs that were biologically active. The RCX is the relative $^{45}Ca^{2+}$ influx, normalized to the level of stimulation produced by the reference $1\alpha,25(OH)_2D_3$ at a concentration of 1 nM, which was set to 100%. The 6-s-*trans* locked analog (**JB**) was unable to act as an agonist, suggesting that the response element (receptor) that is coupled to the signal transduction process stimulating $^{45}Ca^{2+}$ uptake is not responsive to 6-s-*trans* analogs like $1\alpha,25(OH)_2$-tachysterol$_3$. In contrast, all five of the 6-s-*cis* locked analogs (**JC**, **JM**, **JN**, **JO**, **JP**) were effective at stimulating a rapid uptake of $^{45}Ca^{2+}$, although there was some variability in the reproducibility of the RCX values over a series of five separate experiments. The two analogs that were most consistently equivalent in activity to $1\alpha,25(OH)_2D_3$ were analogs **JN** and **JP** (RCX range ~60–100%), while analogs **JM** and **JO** were somewhat less active (RCX range ~40–80%).

Genomic Actions

Figures 4–6 and Table 3 report an evaluation in three cultured cell lines of the ability of the various analogs to initiate biological responses via signal transduction pathways believed to require the participation of the $1\alpha,25(OH)_2D_3$ VDR_{nuc}. $1\alpha,25(OH)_2D_3$ and analogs were evaluated in MG-63 cells for their relative ability to induce human osteocalcin (Fig. 4). Three of the four 6-s-*cis* locked $1\alpha,25(OH)_2$-provitamins (**JM, JO**, **JP**), were found to be 800- to 30,000-fold less effective than $1\alpha,25(OH)_2D_3$ in inducing osteocalcin; the fourth analog, **JN**, was the most effective of the diastereomers; however, it was still only 250-fold less effective than $1\alpha,25(OH)_2D_3$ (see Table 3). These results are consistent with the interpretation that the 6-s-*cis* conformation is not able to efficiently interact with the VDR_{nuc} present in the MG-63 cells.

Figures 5 and 6 report the relative ability of the analogs to interact in two separate systems employing transiently transfected $1\alpha,25(OH)_2D_3$ nuclear receptor response elements (VDREs) linked to reporter genes. $1\alpha,25(OH)_2D_3$-stimulated reporter gene activity is dependent upon the functioning of the VDR_{nuc}. Figure 5 reports the efficacy of the analogs to mediate an induction of GH in transfected COS-7 cells. Both the two 6-s-*trans* analogs (**JB**, **JD**) and the four 6-s-*cis* locked analogs (**JM, JN**, **JO**, **JP**) were found to be relatively less active (42,000- to 100,000-fold) than $1\alpha,25(OH)_2D_3$ in activating the GH promoter (see Table 3). These results suggest that neither 6-s-*trans* (**JB**,

Table 2. Summary of the Relative Ability of $1\alpha,25(OH)_2D_3$ and Conformationally Locked B Ring Analogs to Stimulate Rapid, Nongenomic Biologic Responses

Compound Name	Analog Code	Orientation (6,7 bond)[a]	Transcaltachia (%)	$^{45}Ca^{2+}$ Uptake into ROS 17/2.8 Cells	
				$[Ca^{2+}]^b_{max}$	RCX[c]
$1\alpha,25(OH)_2D_3$	C	*cis* and *trans*	100	1.0×10^{-9}	100
$1\alpha,25(OH)_2$-Tachysterol$_3$	**JB**	*trans*	2	Inactive	Inactive
$1\alpha,25(OH)_2$ cis-Isotachysterol$_3$	**JC**	*cis*	75	5×10^{-9}	100
$1\alpha,25(OH)_2$-trans-Isotachysterol$_3$	**JD**	*trans*	3	NA[d]	NA[c]
$1\alpha,25(OH)_2$-7-Dehydrocholesterol	**JM**	*cis*	60	5×10^{-11}	31–100
$1\alpha,25(OH)_2$-Lumisterol$_3$	**JN**	*cis*	105	(a) 1×10^{-10} (b) 1×10^{-8}	50–100
$1\alpha,25(OH)_2$-Pyrocalciferol$_3$	**JO**	*cis*	50	1×10^{-8}	36–75
$1\alpha,25(OH)_2$-Isopyrocalciferol$_3$	**JP**	*cis*	60	1×10^{-10}	70–100

This table summarizes results for all analogs with regard to their ability to stimulate either transcaltachia or the uptake of $^{45}Ca^{2+}$ in ROS 17/2.8 cells. Typical transcaltachia results are presented in Fig. 2; the results summarized here are the average of two to four perfusions. The mean relative transcaltachic activity for each analog at 40 min, expressed as the percent of the (treated/basal) ratio for $1\alpha,25(OH)_2D_3$. Each analog produced a maximum response between 650–1300 pM (see text). Typical $^{45}Ca^{2+}$ dose response data for analogs **JM** and **JN** are presented in Fig. 3. For the RCX results, the range of results over a series of five experiments are reported.

[a] Description of the orientation around the 6,7 carbon bond of the B ring of the indicated analog in either the 6-s-*cis* or 6-s-*trans* locked conformation.

[b] Concentration of analog stimulating the maximum influx of $^{45}Ca^{2+}$ in to the ROS 17/2.8 cells.

[c] RCX is the relative $^{45}Ca^{2+}$ influx, normalized to the level of stimulation produced by the reference $1\alpha,25(OH)_2D_3$ at a concentration of 1 nM, which is set at a value of 100%.

[d] NA, Not assayed.

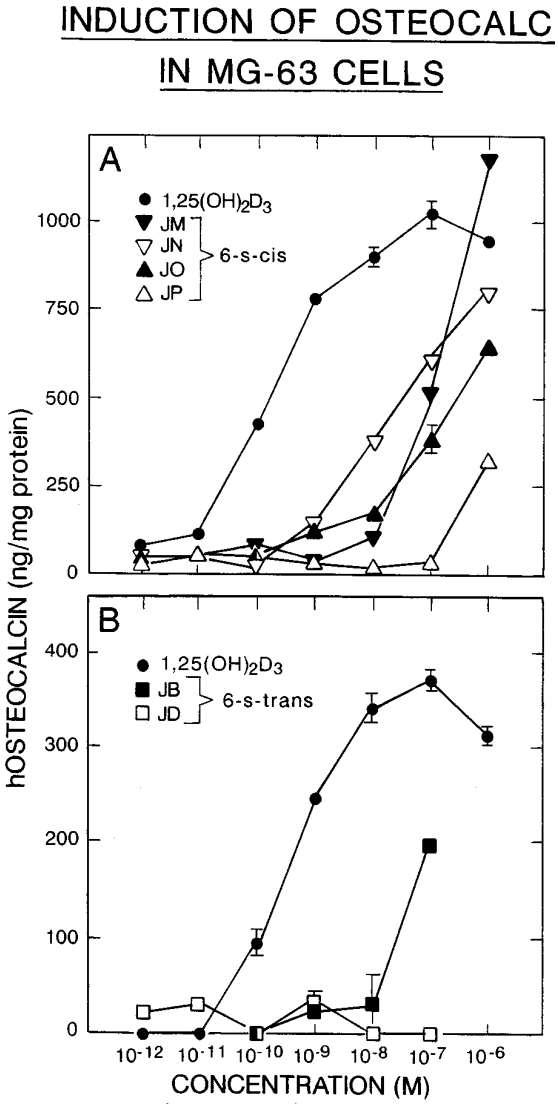

Fig. 4. Efficacy of $1\alpha,25(OH)_2D_3$, 6-s-*cis* Locked $1\alpha,25(OH)_2$-Provitamins D [**JM**, **JN**, **JO**, **JP**] (panel A) and 6-s-*trans* Locked Analogs (**JB**, **JD**) (panel B) to Induce Osteocalcin in MG-63 Cells

For details see *Materials and Methods*. The data presented are from a representative experiment; a total of three separate experiments was conducted. The error bars shown are SEM for triplicate determinations in one experiment. The symbols are defined in the figure panels. A summary is presented in Table 3.

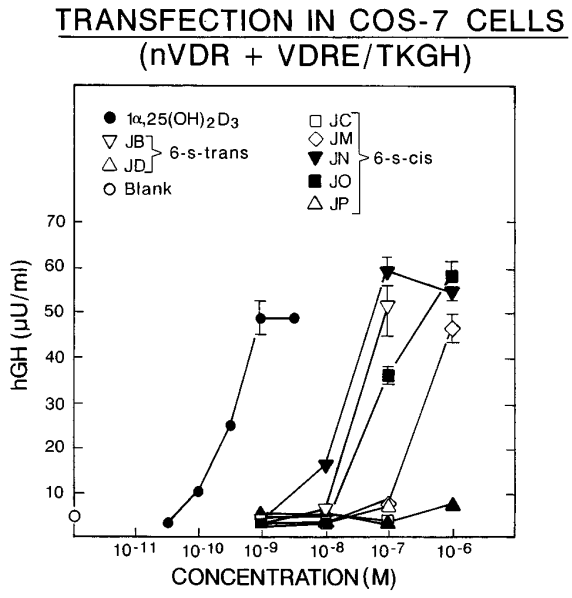

Fig. 5. Efficacy of $1\alpha,25(OH)_2D_3$, 6-s-*cis* Locked $1\alpha,25(OH)_2$-Provitamins D (**JM**, **JN**, **JO**, **JP**) and 6-s-*trans* Locked Analogs (**JB**, **JD**) to Induce GH Expression in Transfected COS-7 Cells

Cells were cotransfected with the pSG5hVDR expression plasmid and the VDRE linked to the reporter plasmid $(CT)_4$TKGH. Next the cells were exposed for 24 h to different concentrations of $1\alpha,25(OH)_2D_3$ or analogs. The medium was assayed for the expression of human GH via RIA. See *Materials and Methods* for additional details. These are the results of a representative experiment that was conducted three times. Where shown the error bars are SEM of triplicate measurements from one experiment. The symbols are defined in the figure panel. The data are summarized in Table 3.

JD) nor 6-s-*cis* locked analogs (**JM, JN**, **JO**, **JP**) are able to interact effectively with the VDR_{nuc} for $1\alpha,25(OH)_2D_3$ that was transfected into the COS-7 cells.

Figure 6 reports the agonist activity of the five 6-s-*cis* $1\alpha,25(OH)_2D_3$ analogs (**JC**, **JM**, **JN**, **JO**, **JP**) and the two 6-s-*trans* locked analogs (**JB**, **JD**) on induction of luciferase activity in ROS 17/2.8 cells after transient transfection of the VDRE containing the 1.7RI-Luc fusion construct. Several reports have clearly documented the ability of $1\alpha,25(OH)_2D_3$ to induce OPN at the level of transcription in ROS 17/2.8 cells, which are known to express high levels of the VDR_{nuc}. With the exception of analog **JN**, all other analogs from both the 6-s-*cis* and 6-s-*trans* families displayed a potency ranging from only 6% to 0.2% of the activity of $1\alpha,25(OH)_2D_3$, for which the measured luciferase activity was set to 100%. The results suggest that neither family of conformationally locked analogs can interact productively with the VDRE present in the osteopontin promoter so as to result in an activation of the luciferase reporter. In contrast, analog **JN** was consistently found to have 28–56% of the activity of $1\alpha,25(OH)_2D_3$; this result is addressed in *Discussion*.

Table 3 presents a summary of the relative ability of $1\alpha,25(OH)_2D_3$ and its B-ring analogs to inhibit cell proliferation or modulate gene expression. The data describing the induction of osteocalcin in MG-63 cells and the induction of hGH in COS-7 cells were presented in Figs. 4 and 5, respectively, while the original data describing the results from induction of nitroblue tetrazolium (NBT) activity in HL-60 cells (evaluated by the appearance of NBT reduction) and inhibition of proliferation of MCF-7 cells (quantitated by inhibition of $[^3H]$thymidine incorporation) are not presented. Collectively, the results obtained for both the HL-60 cells

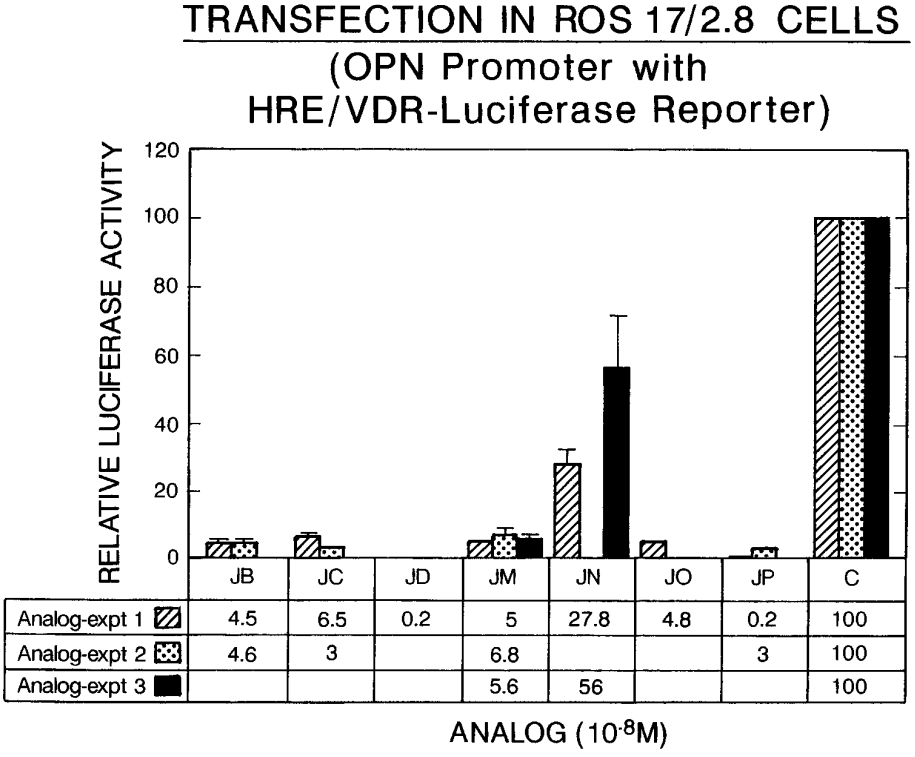

Fig. 6. Efficacy of $1\alpha,25(OH)_2D_3$, 6-s-*cis* Locked $1\alpha,25(OH)_2D_3$ Analogs (**JC**, **JM**, **JN**, **JO**, **JP**) and 6-s-*trans* Locked Analogs (**JB**, **JD**) to Induce Luciferase Activity in Transfected ROS 17/2.8 Cells

The cells were transfected with the 1.7RI-Luc reporter gene construct containing two VDREs. Cells were exposed to $1\alpha,25(OH)_2D_3$ or test analogs at 10 nM for 24 h before assay of luciferase activity. Additional details are provided in *Materials and Methods*. The figure summarizes the relative luciferase activity in relation to the standard $1\alpha,25(OH)_2D_3$ (C) for which the results were set to 100%. The results are expressed as the mean ± SEM of triplicate determinations in a given experiment. The table at the *bottom* of the figures reports results from three experiments.

and MCF-7 cells suggest that neither 6-s-*cis* nor 6-s-*trans* locked analogs are able to interact effectively with the VDR_{nuc} for $1\alpha,25(OH)_2D_3$, which is known to be present [HL-60 cells (40, 41), MCF-7 cells (42, 43)] and associated with the process of cell differentiation.

DISCUSSION

The studies reported in this communication were designed to measure differences in agonist activity of the two analogs representing the 6-s-*trans* (extended steroid) and the five analogs representing the 6-s-*cis* (steroid-like) conformations of $1\alpha,25(OH)_2D_3$ (see Fig. 1A) with respect to initiation of genomic and rapid/nongenomic biological responses. The results are consistent with the model that the genomic and rapid responses are mediated by separate receptors with distinct preferences with regard to the conformation of their agonist ligand. The signal transduction pathway mediating rapid, nongenomic events responds very well to 6-s-*cis* analogs, while the VDR_{nuc} responds poorly to analogs locked into the 6-s-*cis* and is not able to respond to analogs locked in the 6-s-*trans* conformation.

For $1\alpha,25(OH)_2D_3$ the interconversion between the 6-s-*trans* and 6-s-*cis* forms occurs in solution millions of times per second at room temperature and generates a continuum of conformers. As yet, the true equilibrium ratio of the 6-s-*trans* and 6-s-*cis* conformers of any vitamin D seco steroid, including $1\alpha,25(OH)_2D_3$, has not been rigorously determined. It has been estimated by computational methods that 88–99% of $1\alpha,25(OH)_2D_3$ exists as the 6-s-*trans* conformation, with only 1–12% existing in the 6-s-*cis* form. This topic has been reviewed (33), and it seem more reasonable on steric grounds that $1\alpha,25(OH)_2D_3$ is primarily (closer to 99% rather than 88%) in the 6-s-*trans* form. However, a more significant point is that due to the facile interconversion of the 6-s-*trans* and 6-s-*cis* conformers of $1\alpha,25(OH)_2D_3$, there exist kinetically competent amounts of all conformers available to interact with any receptors which may, in turn, be linked to the generation of biological responses. Accordingly, in the absence of direct structural information on protein-bound ligands, insight as to the relative importance of the steroid or extended conformation in the biological actions(s) of $1\alpha,25(OH)_2D_3$ can more practically be obtained through studies of locked analogs.

Table 3. Summary of the Relative Ability of $1\alpha,25(OH)_2D_3$ and Conformationally Locked B ring Analogs to Stimulate Biologic Responses Dependent upon Gene Expression

Compound Name	Analog Code	Orientation (6,7 bond)[a]	Induction of NBT (HL-60[b] cells)	Inhibition of Proliferation (MCF-7[b] cells)	Induction of osteocalcin (MG-63[b] cells)	Induction of hGH (COS-7 cells[b])
$1\alpha,25(OH)_2D_3$	C	*cis* and *trans*				
$1\alpha,25(OH)$-Tachysterol$_3$	**JB**	*trans*	NA	NA	400	73
$1\alpha,25(OH)_2$-trans-Isotachysterol$_3$	**JD**	*trans*	>1000	>1000	>1000	>100,000
$1\alpha,25(OH)_2$-7-Dehydrocholesterol	**JM**	*cis*	1000	NA	830	730
$1\alpha,25(OH)_2$-Lumisterol$_3$	**JN**	*cis*	50	130	250	42
$1\alpha,25(OH)_2$-Pyrocalciferol$_3$	**JO**	*cis*	>1000	130	2000	130
$1\alpha,25(OH)_2$-Isopyrocalciferol$_3$	**JP**	*cis*	>1000	>1000	30,000	>100,000

[a] Description of the orientation around the 6,7 carbon bond of the B ring of the indicated analog in either the 6-s-*cis* or 6-s-*trans* conformation.

[b] The data for each analog are presented as "fold less effective than $1\alpha,25(OH)_2D_3$" from evaluation in four separate assays. In HL-60, cell differentiation was assessed by induction of NBT. In MCF-7 cells inhibition of proliferation was determined via measurement of incorporation of [^{3}H]thymidine. In MG-63 cells the induction of osteocalcin was determined via RIA. The COS cells were cotransfected with the pSG5hVVDR expression plasmid and the $1\alpha,25(OH)_2D_3$ responsive-element (VDRE), which contained the reporter plasmid (CT4)4TKGH. The induction of human GH was measured via RIA. The data are calculated from information presented in Figs. 4 and 5 (or in data not presented for the HL-60 and MCF-7 cells) and represent the ratio of [ED_{50} of the analog]/[ED_{50} for $1\alpha,25(OH)_2D_3$]. NA, Not assayed. For all results the data represent the mean of at least three experiments.

This present study employed two classes of analogs of $1\alpha,25(OH)_2D_3$ which are locked in a defined conformation. One class consists of four diastereomers of the provitamin D form of $1\alpha,25(OH)_2D_3$ (**JM**, **JN**, **JO**, **JP**). Because these analogs are not seco steroids, *i.e*. their 9,10 carbon-carbon bond is not broken, they are permanently locked in the 6-s-*cis* conformation (see Fig. 1C) and as ligands for receptors there is only one conformation present in rings A, B, C, and D. The two relevant asymmetric centers, which are at carbon-9 and carbon-10, result in four analogs (diastereomers) each with an α or β orientation of the hydrogen on C-9 and the methyl group on C-10.

The second class of analogs employed in our studies consists of three seco steroids with a double bond between carbons 6 and 7 (see Fig. 1B); accordingly, there can be no rotation around the 6,7 single bond as in $1\alpha,25(OH)_2D_3$. Thus, analogs **JB** and **JD** are 6-s-*trans* locked analogs, while analog **JC** is a 6-s-*cis* locked analog. However it should be appreciated that **JB**, **JC**, and **JD** do display conformational mobility around their two 5,6 and 7,8 single carbon bonds, which permits them to generate a population of conformations not available to $1\alpha,25(OH)_2D_3$.

The uniformity of the inability of both the 6-s-*cis* and 6-s-*trans* locked analogs to act as an agonist for the six genomic assays is impressive. None of the analogs had a significant affinity for the VDR_{nuc} (Table 1). In addition, the lack of genomic responses was not limited to one cell type or system; there was no detectable genomic effect at physiological concentrations of the analogs *in vivo* in the vitamin D-deficient chick (Table 1), in HL-60, or MCF-7 cells (Table 3), or in COS-7 (Fig. 5 and Table 3) or ROS 17/2.8 cells (Fig. 6), which had been transfected with a promoter containing the VDREs for $1\alpha,25(OH)_2D_3$. These results suggest that the VDR_{nuc} utilizes a $1\alpha,25(OH)_2D_3$ ligand conformation that is not provided by the conformation of either the 6-s-*cis* or 6-s-*trans* locked analogs employed in these studies.

Our results demonstrate that two rapid nongenomic biological systems are fully responsive to the group of 6-s-*cis* analogs. Both the process of transcaltachia, as studied in the isolated perfused chick duodenum (Fig. 2) (7–9), and the process of Ca^{2+} channel opening in the rat osteogenic sarcoma cell line (12–14) and $^{45}Ca^{2+}$ uptake in ROS 17/2.8 cells (Fig. 3 and Table 2) (15) respond with approximately equivalent potency to the 6-s-*cis* analogs. Interestingly though, not all four $1\alpha,25(OH)_2$-provitamin D_3 diastereomers were 100% as active as $1\alpha,25(OH)_2D_3$, which no doubt reflects the fact that there are subtle differences in the shape of this molecule resulting from different α or β orientations of the hydrogen on C-9 and the methyl group on C-10 (see Fig. 1). The relative rank order of potency for transcaltachia (Fig. 2) was **JN** > **JM** ~ **JP** > **JO**, while the rank order of potency for $^{45}Ca^{2+}$in the ROS 17/2.8 cells (Fig. 3 and Table 2) was **JN** ~ **JP** > **JM** > **JO**. These results are also noteworthy in that this represents the first clear demonstration that 6-s-*cis* locked provitamins D are capable of being potent mediators of selected vitamin D responses. Before this observation it was generally believed that only vitamin D seco steroids could generate biological responses (1, 44).

For one 6-s-*cis* locked analog (**JN**) there was some evidence in ROS 17/2.8 cells of its ability to activate over 24 h a luciferase reporter linked to the OPN hormone-response element for VDR_{nuc} (Fig. 6). In light of the inability of **JN** to consistently activate four other genomic assays (Table 3) and its very weak ability to bind to the VDR_{nuc} under *in vitro* conditions (RCI = 1.8%; see Table 1), the mechanism by which the nuclear activation of the OPN promoter occurs is open to speculation. Bhatia *et al* (27) have recently reported

that NB4 promyelocytic leukemic cells could be stimulated to differentiate into macrophages by combination treatment with a 6-s-*cis* locked analog, $1\alpha,25(OH)_2$-d_5-pre-D_3 (**HF**), and phorbol ester; these authors proposed that analog **HF** was able to interact with a putative membrane receptor that engaged in cross-talk in collaboration with phorbol ester to effect the onset of the nuclear response of cell differentiation. Our previous studies have clearly established that **HF** is not able to bind effectively to the VDR_{nuc} or to initiate genomic responses (34). Thus it is possible that in the ROS 17/2.8 cells, where there is evidence for both a VDR_{nuc} and putative membrane receptor (16, 39, 45), analog **JN** may interact with the putative membrane receptor, which via signal transduction cross-talk pathways, then results in the activation of the nuclear response of induction of the mRNA for OPN by VDR_{nuc} independent pathways. Consistent with this suggestion are recent results from this laboratory that indicate that 6-s-*cis* locked, but not 6-s-*trans* locked, analogs (**JN** *vs.* **JB**) can stimulate mitogen-activated protein kinases in both chick intestinal cells (46) and NB4 cells (47). Mitogen-activated protein kinases are known to be able to integrate multiple intracellular signals transmitted by various second messengers so as to regulate many cellular functions by phosphorylation of numbers of cytoplasm kinases and nuclear transcription factors including the epidermal growth factor receptor, c-Myc, and c-Jun (48). Another alternative explanation might be that analog **JN** or **JO** is metabolized into seco steroids that have a more favorable interaction with the VDR_{nuc}; however, this seems unlikely as there are no known lyase enzymes that can break the 9,10 carbon bond of $1\alpha,25(OH)_2$-7-dehydrocholesterol.

The responsiveness of the signal transduction process for the two rapid systems occurs in two species, the rat and chick, and in two different vitamin D target organs, the intestine and bone. In both systems there is evidence that the biological response involves the opening of voltage-sensitive Ca^{2+} channels that are located in the outer cell membrane (12–14). It has been postulated for both systems that the signal transduction process that results in the opening of the Ca^{2+} channel may involve a putative membrane receptor for $1\alpha,25(OH)_2D_3$. Biochemical evidence has been presented for the existence of a binding protein specific for $1\alpha,25(OH)_2D_3$ present in the chick intestinal basal lateral membrane that is correlated with the process of transcaltachia; this protein has been purified 4500 fold [dissociation constant (K_D) = 0.72×10^{-9} M for $1\alpha,25(OH)_2D_3$] (49).

In both the ROS 17/2.8 cell system and in the perfused intestinal transcaltachic system, an evaluation has been made of a series of analogs with differing structural modifications of the reference compound, $1\alpha,25(OH)_2D_3$. Evidence was obtained for two classes of analogs, those which bind effectively to the $1\alpha,25(OH)_2D_3$ nuclear receptor but which are ineffective at opening Ca^{2+} channels and those analogs which are effective in stimulating the opening of Ca^{2+} channels but which bind poorly to the $1\alpha,25(OH)_2D_3$ nuclear receptor (9, 15, 39, 45, 50). Collectively, these results have been interpreted as suggesting the existence of two forms of the $1\alpha,25(OH)_2D_3$ receptor, one present in the nucleus/cytosol concerned with genomic responses and a second species present in the plasma membranes of some cells, which are involved in some fashion with rapid nongenomic biological responses related to $1\alpha,25(OH)_2D_3$. Further support for two receptors for 1,25-$(OH)_2D_3$ has come from studies with $1\beta,25(OH)_2D_3$. This analog binds very poorly to the nuclear $1\alpha,25(OH)_2D_3$ receptor (RCI = 0.01), and although it is devoid of agonist activity in transcaltachia, it has been found that $1\beta,25(OH)_2D_3$ is a potent antagonist of both $1\alpha,25(OH)_2D_3$-stimulated transcaltachia (51, 52) and the opening of L-type Ca^{2+} channels (14).

The principal carrier of vitamin D seco steroids throughout the body is the plasma vitamin D-binding protein (DBP). This protein, which has a ligand binding domain for vitamin D seco steroids, transports vitamin D_3, $25(OH)D_3$, 1,25-$(OH)_2D_3$, and 24R,$25(OH)_2D_3$ throughout the plasma compartment (53, 54). It is also of importance to learn whether the ligand-binding domain of DBP prefers the steroid-like conformation (6-s-*cis* conformer) or the extended steroid conformation (6-s-*trans* conformer). When the data of Table 1 are evaluated, it is clear that the four $1\alpha,25(OH)_2$-provitamins D_3 (6-s-*cis* locked) and analogs **JB** and **JD** (6-s-*trans* locked) all had very low RCI values in comparison to the reference 1,25-$(OH)_2D_3$, whose RCI is 100%. Thus we tentatively conclude that the ligand-binding domain of DBP prefers neither the 6-s-cis steroid-like conformation nor that of the 6-s-trans extended steroid conformation of 1,25-$(OH)_2D_3$.

On the basis of the data presented in this communication, we conclude that the generation of rapid biological responses prefers ligands that are 6-s-*cis* locked rather than ligands that are 6-s-*trans* locked. In contrast to our positive conclusion relative to the rapid responses, we have gained indirect insight only into the preferred ligand configurations for the VDR_{nuc}. Based on the seven analogs studied to date, we can only conclude that the VDR_{nuc} does not prefer analogs that are either 6-s-*trans* or 6-s-*cis* locked. It remains to the future to synthesize conformationally locked analogs that are restrained in intermediate conformations relative to the extreme limits defined by the 6-s-*cis* and 6-s-*trans* locked analogs. Also it is possible that the VDR_{nuc} requires that its optimal ligands retain conformational flexibility about the 6,7 single carbon bond in yet some unknown fashion.

We are currently synthesizing additional analogs of $1\alpha,25(OH)_2D_3$ and conducting further studies to explore these various possibilities. Collectively these studies demonstrate the complexity of the structure-function relationships in the vitamin D endocrine system and the ligand-binding domain of the $1\alpha,25(OH)_2D_3$ receptors and binding proteins.

MATERIALS and METHODS

Chemicals

$^{45}CaCl_2$ was obtained from New England Nuclear (Boston, MA). 1,25-$(OH)_2D_3$ was the kind gift of Dr. Milan Uskokovic (Hoffmann La Roche, Nutley, NJ). [methyl-^{3}H]Thymidine (2 Ci/mmol) was purchased from Amersham (Buckinghamshire, U.K.). Cell culture media were purchased from GIBCO (Roskilde, Denmark). Penicillin and streptomycin were from Boehringer (Mannheim, Germany). NBT was obtained from Sigma (St. Louis, MO).

Chemical Synthesis of Four 6-s-*cis* 1α,25$(OH)_2$-Provitamins D (Analogs JM, JM, JO, and JP) and the Two 6-s-trans Analogs, 1α,25(OH)-Dihydrotachysterol$_3$ (JB) and 1α,25-$(OH)_2$-trans-Isotachysterol$_3$ (JD)

Figure 1 presents the structure of all the analogs of 1α,25$(OH)_2D_3$ used in this study. Presented below is a description of the chemical synthesis of the four 6-s-*cis* locked diastereomers.

1α,25-Dihydroxytachysterol (Analog JB) A sample of 1α,25-dihydroxyprevitamin D_3 (15 mg, 0.0360 mmol) in ether (2 ml) was isomerized with iodine in ether (0.49 mM, 300 μl) using a standard procedure (55). HPLC (Rainin Microsorb, Rainin Instrument Co., Woburn, MA; 5 μm silica, 10 mm $\times$ 25 cm, 11% isopropanol/hexanes) afforded tachysterol analog **JB** (8 mg, 53%). ^{1}*H-NMR*: ($CDCl_3$) δ 0.69 (3H, C_{18}-CH_3, s), 0.98 (3H, C_{21}-CH_3, d, J$\sim$6.4 Hz), 1.22 (6H, $C_{26,27}$-CH_3, s), 1.91 (3H, C_{19}-CH_3, s, 4.14 (1H, H_3, dddd, J$\sim$9.5 Hz, 9.5 Hz, 4.7 Hz, 4.7 Hz), 4.24 (1H, H_1, br s), 5.75 (1H, H_9, br s), 6.15 (1H, $H_{6\ or\ 7}$, d, J$\sim$16.2 Hz), 6.65 (1H, $H_{7\ or\ 6}$, d, J$\sim$16.2 Hz). *UV*: (100% EtOH) λ_{max} 282 nm (ϵ 20, 300); λ_{sh} 290 nm (ϵ 18, 100), 272 nm (ϵ 16, 800). *HRMS*: m/z 416.3283 (calculated for $C_{27}H_{44}O_3$, 416.3290).

1α,25-Dihydroxy-cis-isotachysterol (Analog JC) and 1α,25-Dihydroxyisotachysterol (Analog JD) Analog **JC** was prepared as previously reported by VanAlstyne *et al.* (56). A sample of **JC** (7.5 mg, 0.0180 mmol) in ether (1 ml) was isomerized in ether (0.49 mM, 150 μl) using the standard procedure above (55). HPLC (as above for **JB)** afforded in order of elution starting **JC** (2.1 mg, 28%) and analog **JD** (3.9 mg, 52%). Data for **JD**: ^{1}*H-NMR:* ($CDCl_3$) δ 0.91 (3H, C_{18}-CH_3, s), 0.97 (3H, C_{21}-CH_3, d, J$\sim$6.6 Hz), 1.22 (6H, $C_{26,27}$-CH_3, s), 1.92 (3H, C_{19}-CH_3, s), 1.0–2.8, 4.16 (1H, H_3, m), 4.25 (1H, H_1, br s), 6.47 (1H, $H_{7\ or\ 6}$, d, J$\sim$16.0 Hz), 6.54 (1H, $H_{7\ or\ 6}$, d, J$\sim$16.0 Hz). *UV*: (100% EtOH) λ_{max} 292 nm (ϵ 30, 800); λ_{sh} 302 nm (ϵ 23, 100), 282 nm (ϵ 25, 600). *HRMS*: (FAB, Et_2O/NBA) m/z 416.3302 (calculated for $C_{27}H_{44}O_3$, 417.3292).

1α,25-Dihydroxy-7-dehydrocholesterol (Analog JM, 9α, 10β-Isomer) and 1α,25-Dihydroxylumisterol (Analog JN, 9β, 10α-Isomer) After 1α,25-dihydroxyprevitamin D_3 (120 mg) (55) in methanol was photolyzed (Hanovia 450 watt medium pressure mercury lamp (Engelhard Hanovia Inc., Newark, NJ), pyrex filter, $\lambda > 300$ nm) for 3 h at room temperature the residue after concentration was subjected to HPLC to afford in order of elution **JM** (9.1 mg, 7.6%), **JN** (15.0 mg, 12.5%), and the starting previtamin (10.6 mg, 8.8%). Analysis of the crude mixture by ^{1}H-NMR spectroscopy showed the ratio of **JN:JM** to be 3:1. Data for **JM**: ^{1}*H-NMR*: ($CDCl_3$) δ 0.63 (3H, C_{18}-CH_3, s), 0.95 (3H, C_{19}-CH_3, s), 0.96 (3H, C_{21}-CH_3, d, J$\sim$5.6 Hz), 1.22 (6H, $C_{26,27}$-CH_3, s), 2.70 (1H, m), 3.77 (1H, H_1, br s), 4.07 (1H, H_3, m), 5.38 (1H, $H_{6\ or\ 7}$, ddd, J$\sim$5.5 Hz, 2.8 Hz, 2.8 Hz), 5.73 (1H, $H_{7\ or\ 6}$, dd, J$\sim$5.5 Hz, 2.2 Hz). *UV*: (100% EtOH) λ_{max} 294 nm (ϵ 8, 400), 282 nm (ϵ 13, 400), 272 nm (ϵ 12, 800); λ_{min} 290 nm (ϵ 7, 800), 278 nm (ϵ 11, 500); l_{sh} 264 nm (ϵ 9, 600). *HRMS*: (CI, CH_4) m/z 417.3365 (calcd. for $C_{27}H_{44}O_3$ plus H, 417.3370 Data for **JN**: ^{1}*H-NMR*: ($CDCl_3$) δ 0.61 (3H, C_{18}-CH_3, s), 0.78 (3H, C_{19}-CH_3, s), 0.91 (3H, C_{21}-CH_3, d, J$\sim$5.2 Hz), 1.21 (6H, $C_{26,27}$-CH_3, s), 4.10 (1H, H_1, dd, J$\sim$9.2 Hz, 4.8 Hz), 4.14 (1H, H_3, dd, J$\sim$3.0 Hz, 3.0 Hz), 5.45 (1H, $H_{6\ or\ 7}$, m), 5.75 (1H, $H_{7\ or\ 6}$, dd, J$\sim$5.1 Hz, 1.7 Hz). *UV*: (100% EtOH) λ_{max} 282 nm (ϵ 6, 900), 274 nm (ϵ 7, 300); λ_{sh} 294 nm (ϵ 3, 900), 264 nm (ϵ 5, 900). *HRMS*: m/z (CI, CH_4) 417.3365 (calculated for $C_{27}H_{44}O_3$ plus H, 417.3370).

1α,25-Dihydroxypyrocholecalciferol (Analog JO, 9α, 10α-Isomer) and 1α,25-Dihydroxyisopyrocholecalciferol (Analog JP, 9β, 10β-Isomer) An argon flushed solution of 1α,25-dihydroxyprevitamin D_3 (54.2 mg) dissolved in DMF (15 ml) containing one drop of 2,4,6-trimethylpyridine was heated in a screw cap vial (156 C) for 18 h. After concentration the crude residue was purified by HPLC (as above) to afford in order of elution analog **JP** (7.3 mg, 13.5%), analog **JO** (20.1 mg, 37.1%), and 1α,25-dihydroxyvitamin D_3 (2.1 mg, 3.9%). Analysis of the crude mixture by ^{1}H-NMR spectroscopy showed the ratio of **JO** to **JP** to be 3:1. Data for **JO**: ^{1}*H-NMR:* ($CDCl_3$) δ 0.53 (3H, C_{18}-CH_3, s), 0.90 (3H, C_{21}-CH_3, d, J$\sim$6.0 Hz), 1.02 (3H, C_{19}-CH_3, s), 1.21 (6H, $C_{26,27}$-CH_3, s), 0.80–2.05, 2.15 (1H, dd, J$\sim$12.6 Hz, 7.6 Hz), 2.26 (1H, d with fine structure, J$\sim$6.1 Hz), 2.54 (1H, br, d, J$\sim$6.1 Hz), 4.16 (1H, H_3, dddd, J$\sim$2.8 Hz, 2.8 Hz, 2.8 Hz, 2.8 Hz), 4.31 (1H, H_1, dd, J$\sim$12.0 Hz, 4.6 Hz), 5.34 (1H, $H_{6\ or\ 7}$, d, J$\sim$5.7 Hz), 5.61 (1H, $H_{7\ or\ 6}$, dd, J$\sim$5.7 Hz, 2.5 Hz). *UV*: (100% EtOH) λ_{max} 286 nm (ϵ 9, 400), 276 nm (ϵ 9, 300); λ_{min} 280 nm (ϵ 8, 800); λ_{sh} 296 nm (ϵ 5, 700), 266 nm (ϵ 7,000). *HRMS*: (CI, CH_4) m/z 417.3366 (calcd. for $C_{27}H_{44}O_3$ plus H, 417.3370). Data for **JP**: ^{1}*H-NMR*: ($CDCl_3$) d 0.65 (3H, C_{18}-CH_3, s), 0.92 (3H, C_{21}-CH_3, d, J$\sim$5.3 Hz), 1.21 (6H, $C_{26,\ 27}$-CH_3, s), 1.30 (3H, C_{19}-CH_3, s), 0.80–2.), 3.71 (1H, H_1, dd, J$\sim$2.8 Hz, 2.8 Hz), 3.94 (1H, H_3, dddd, J$\sim$10.9 Hz, 10.9 Hz, 5.5 Hz, 5.5 Hz), 5.34 (1H, $H_{6\ or\ 7}$, ddd, J$\sim$5.5 Hz, 2.7 Hz, 2.7 Hz), 5.95 (1H, $H_{7\ or\ 6}$, d, J$\sim$5.5 Hz). *UV*: (100% EtOH) λ_{max} 286 nm (ϵ 7, 800), 278 nm (ϵ 7, 900); λ_{sh} 296 nm (ϵ 5, 200), 270 nm (ϵ 6, 500). *HRMS*: (CI, CH_4) m/z 417.3351 (calcd. for $C_{27}H_{44}O_3$ plus H, 417.3370).

Animals and Cells

Riverside White Leghorn cockerels (Hyline International, Lakeview, CA) were obtained on the day of hatch and maintained on a vitamin D-supplemented diet (1.0% calcium and 1.0% phosphorus; O. H. Kruse Grain and Milling, Ontario, CA) for 5–6 weeks to prepare normal vitamin D_3-replete chicks for use in the transcaltachia studies. All experiments employing animals were approved by the University of California-Riverside Chancellor's Committee on Animals in Research.

Leuven The human promyelocytic leukemia cell line (HL-60), the MCF-7 cell line, the COS-7 cell line, and the MG-63 cells were obtained from the American Type Culture Collection (Rockville, MD).

Houston The ROS 17/2.8 cells (kindly provided originally by Dr. Gideon Rodan, Merck, Sharp and Dohme, West Point, PA) were cultured in DMEM-Ham's F-12 1:1 medium containing 10% FCS (GIBCO BRL, Gaithersburg, MD). For $^{45}Ca^{2+}$ uptake experiments, cells were seeded at a density of 30,000 cells/ml into 3.5-cm dishes and grown to approximately 50% confluency.

Calcium Uptake Assays

ROS 17/2.8 cells were assayed for Ca^{2+} uptake using procedures described previously (16, 34).

Intestinal $^{45}Ca^{2+}$ Transport (Transcaltachia)

Measurements of $^{45}Ca^{2+}$ transport were carried out in perfused chick duodena as previously described (8, 9, 34). In brief, normal vitamin D-replete chicks weighing approximately 500 g were anesthetized with Chloropent (Fort Dodge, IA; 0.3 ml per 100 g), and the duodenal loop was surgically exposed. After cannulation of the celiac artery and vein, the duodena was perfused with modified Grey's balanced salt

solution (GBSS) + 0.9 mM Ca^{2+}which was oxygenated with 95% O_2 and 5% CO_2. A basal transport rate was established by perfusion with control medium for 20 min after the intestinal lumen was filled with $^{45}Ca^{2+}$. The tissue was then exposed to 1,25-$(OH)_2D_3$ or analogs or reexposed to control medium for an additional 40 min. The vascular perfusate was collected at 2-min intervals during the last 10 min of the basal and during the entire treatment period. Duplicate 100-μl aliquots were taken for determination of the $^{45}Ca^{2+}$ levels by liquid scintillation spectrometry. The results are expressed as the ratio of the $^{45}Ca^{2+}$ appearing in the 40-min test period over the average initial basal transport period.

Ligand-Binding Studies

The relative ability of each analog to compete with [^{3}H]1,25-$(OH)_2D_3$ for binding to either the intestinal nuclear receptor for 1,25-$(OH)_2D_3$ from vitamin D-deficient chicks or from a vitamin D-replete pig was carried out under *in vitro* conditions according to our standard procedures (36, 57).

The data was plotted as [competitor]/[[^{3}H]$1\alpha,25(OH)_2D_3$] *vs.* 1/[fraction bound]. The relative competitive index or RCI was calculated as [slope of competitor]/[slope for $1\alpha,25(OH)_2D_3$] $\times$ 100 as previously described (36); such plots give linear curves characteristic for each analog, the slopes of which are equal to the analog's competitive index value (57). The competitive index value for each analog is then normalized to a standard curve obtained with nonradioactive $1\alpha,25(OH)_2D_3$ as the competing steroid and placed on a linear scale of relative competitive index (RCI), where the RCI of $1\alpha,25(OH)_2D_3$ by definition is 100.

The relative ability of each analog to compete with [^{3}H]25(OH)D_3 for binding to the human vitamin D-binding protein was run using human DBP (Gc-Globulin, Sigma, St. Louis, MO) as the binding protein according to our standard procedures (36, 57). The data were plotted as [competitor]/[[^{3}H]25(OH)D_3] *vs.* 1/[fraction bound]. The relative competitive index or RCI was calculated as [slope of competitor]/[slope for 25(OH)D_3] $\times$ 100. Note that although each analog was assayed in competition with [^{3}H]25(OH)D_3, the data are expressed as relative to the binding of $1\alpha,25(OH)_2D_3$, with its RCI set to 100. Thus, when the RCI of $1\alpha,25(OH)_2D_3 = 100$, the RCI for 25(OH)$D_3$ = 66,700.

Culture Conditions for HL-60, MCF-7, COS-7, and MG-63 Cells

HL-60 cells were seeded at 1.2×10^5 cells/ml, and 1,25-$(OH)_2D_3$ or its analogs were added in ethanol (final concentration <0.2%) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (GIBCO), 100 U/ml penicillin, and 100 U/ml of streptomycin (Boehringer). After 4 days of culture in a humidified atmosphere of 5% CO_2 in air at 37 C, the dishes were shaken to loosen any adherent cells, and all cells were then assayed for differentiation by NBT reduction assay and for proliferation by [^{3}H]thymidine incorporation.

The COS-7 cells in Dulbecco's medium supplemented with 10% FCS were seeded into six-well plates to reach 40–60% confluence. After 24 h the medium was removed and refreshed with culture medium containing 2% dextran-coated charcoal-treated FCS. The cells were then cotransfected with the pSG5hVDR expression plasmid (1.5 μg) and the $1\alpha,25(OH)_2D_3$ responsive element (VDRE) linked to the reporter plasmid $(CT4)_4$TKGH (1.5 μg). Finally the cells were exposed to different concentrations of $1\alpha,25(OH)_2D_3$ or analogs. The medium was assayed for the expression of human GH using an in house RIA.

MCF-7 cells were cultured in Dulbecco's MEM nutrient mix F-12 (HAM) medium supplemented with 10% heat-inactivated FCS, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2 in air. MCF-7 cells were seeded at 5000 cells/well in the above described medium in a 96-well microtiter plate in a final volume of 0.2 ml per well. Triplicate cultures were performed. After 24 h, $1\alpha,25(OH)_2D_3$ or analogs were added in the appropriate concentrations for an incubation period of 72 h. Then 1 μCi of [^{3}H]thymidine was added to each well, and the cells were harvested after a 4-h incubation with a Packard harvester and measured by the Packard Topcount System (Packard, Meriden, CT).

The MG-63 cells were seeded at 5×10^3 cells/ml in 96-well flat bottomed culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) in a volume of 200 μl of DMEM containing 2% of heat-inactivated charcoal-treated FCS and 1,25-$(OH)_2D_3$ or its analogs were added in ethanol (final concentration <0.2%). After 72 h of culture in a humidified atmosphere of 5% CO_2 in air at 37 C the inhibition of proliferation was measured by [^{3}H]thymidine incorporation and osteocalcin concentration in the medium was determined using a homologous human RIA (34).

NBT Reduction Assay

Superoxide production was assayed by NBT-reducing activity as described previously (34).

Complimentary DNA Probes and Northern Blot Analyses

Total RNA was extracted from cell monolayers essentially as previously described (39).

Plasmid Construction and Transfection of ROS 17/2.8 Cells

The rat OPN gene and 5′-flanking sequences have been previously isolated and sequenced. Construction of the 1.7RI-Luc reporter gene construct has been described previously (58). Sequence analysis, transient transfection functional assays, and gel shifting assays have confirmed that the 1.7RI fragment derived from the upstream region of the rat OPN gene contains a proximal and a distal VDRE. Both VDREs have been shown to bind the VDR-retinoid X receptor heterodimer, and their composite response to $1\alpha,25(OH)_2D_3$ stimulation is additive (A. L. Ridall *et al.*, manuscript in preparation). Transient transfection in ROS 17/2.8 cells was performed as described previously (16), with the exception that the β-galactosidase activity (monitored for transfection efficiency) was assayed by using AMPGD or (3-(4-methoxyspiro-[1, 2-dioxethane-3.2′-tricyclo-[3.3.1.1^{3,7}]decan]-4-yl)-phenyl-β-D-galactopyranoside) as a substrate, purchased as LumiGal (CLONTECH Laboratories, Inc., Palo Alto, CA). Enzyme activity was monitored on a Turner Td-20 luminometer (Turner Designs, Inc., Mt. View, CA).

Statistics

Statistical evaluation of the data was performed by Students' *t* test for unpaired observations.

Acknowledgments

A.W.N. thanks Ms. Marian Herbert for assistance in preparation of the manuscript. M.C.F.-C. thanks Dr. Jean Weber for assistance with the $^{45}Ca^{2+}$ flux assays. H.vB. and R.B. thank Dr. Mark Haussler for the expression and reporter plasmids used in the COS-7 transfection studies. W.H.O. and M.W.H. thank Ms. Ellen Van Alstyne for samples of $1\alpha,25(OH)_2$-*cis*-Isotachysterol$_3$ (analog **JC**).

Received March 21, 1997. Re-revision received June 13, 1997. Accepted June 17, 1997.

Address requests for reprints to: Anthony W. Norman, Department of Biochemistry, University of California, Riverside, California 92521.

This work was supported in part by UPHS Grant DK-09012–30 (to A.W.N.) and DK-16595 (to W.H.O.), USPHS Grant DE-10318–01 (to M.C.F.-C.), and the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek FGWO 3.0091.93 (to R.B.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*This paper is dedicated to the memory of Murray Carl Dormanen who passed away in September of 1995.

REFERENCES

1. Reichel H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl J Med 320:980–991
2. Bouillon R, Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257
3. Hannah SS, Norman AW 1994 1α,25 $(OH)_2$ vitamin D_3-regulated expression of the eukaryotic genome. Nutr Rev 52:376–382
4. Lian JB, Stein GS 1992 Transcriptional control of vitamin D-regulated proteins. J Cell Biochem 49:37–45
5. Lowe KE, Maiyar AC, Norman AW 1992 Vitamin D-mediated gene expression. Crit Rev Eukar Gene Exp 2:65–109
6. Ribeiro RCJ, Kushner PJ, Baxter JD 1995 The nuclear hormone receptor gene superfamily. Annu Rev Med 46:443–453
7. Nemere I, Yoshimoto Y, Norman AW 1984 Studies on the mode of action of calciferol. LIV. Calcium transport in perfused duodena from normal chicks: enhancement with 14 minutes of exposure to 1α,25-dihydroxyvitamin D_3. Endocrinology 115:1476–1483
8. Yoshimoto Y, Nemere I, Norman AW 1986 Hypercalcemia inhibits the "rapid" stimulatory effect on calcium transport in perfused duodena from normal chicks mediated *in vitro* by 1,25-dihydroxyvitamin D_3. Endocrinology 118:2300–2304
9. Zhou L-X, Nemere I, Norman AW 1992 1,25$(OH)_2$-Vitamin D_3 analog structure-function assessment of the rapid stimulation of intestinal calcium absorption (transcaltachia). J Bone Miner Res 7:457–463
10. De Boland AR, Nemere I, Norman AW 1990 Ca^{2+}-channel agonist Bay K8644 mimics 1,25$(OH)_2$-vitamin D_3 rapid enhancement of Ca^{2+} transport in chick perfused duodenum. Biochem Biophys Res Commun 166:217–222
11. De Boland AR, Norman AW 1990 Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D_3-dependent transcaltachia (the rapid stimulation of duodenal Ca^{2+} transport). Endocrinology 127:2475–2480
12. Yukihiro S, Posner GH, Guggino SE 1994 Vitamin D_3 analogs stimulate calcium currents in rat osteosarcoma cells. J Biol Chem 269:23889–23893
13. Caffrey JM, Farach-Carson MC 1989 Vitamin D_3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. J Biol Chem 264:20265–20274
14. Zanello LP, Norman AW 1996 1α,25$(OH)_2$ vitamin D_3-mediated stimulation of outward anionic currents in osteoblast-like ROS 17/2.8 cells. Biochem Biophys Res Commun 225:551–556
15. Farach-Carson MC, Sergeev IN, Norman AW 1991 Non-genomic actions of 1,25-dihydroxyvitamin D_3 in rat osteosarcoma cells: structure-function studies using ligand analogs. Endocrinology 129:1876–1884
16. Khoury R, Ridall AL, Norman AW, Farach-Carson MC 1994 Target gene activation by 1,25-dihydroxyvitamin D_3 in osteosarcoma cells is independent of calcium influx. Endocrinology 135:2446–2453
17. Sergeev IN, Rhoten WB 1995 1,25-dihydroxyvitamin D_3 evokes oscillations of intracellular calcium in a pancreatic β-cell line. Endocrinology 136:2852–2861
18. Lieberherr M, Grosse B, Duchambon P, Drüke T 1989 A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D_3 on the phosphoinositide metabolism in rat enterocytes. J Biol Chem 264:20403–20406
19. Baran DT, Sorensen AM, Honeyman TW, Ray R, Holick MF 1989 Rapid actions of 1α,25-dihydroxyvitamin D_3 and calcium and phospholipids in isolated rat liver nuclei. FEBS Lett 259:205–208
20. Bourdeau A, Atmani F, Grosse B, Lieberherr M 1990 Rapid effects of 1,25-dihydroxyvitamin D_3 and extracellular Ca^{2+} on phospholipid metabolism in dispersed porcine parathyroid cells. Endocrinology 127:2738–2743
21. Tsutsumi M, Alvarez U, Avioli LV, Hruska KA 1985 Effect of 1,25-dihydroxyvitamin-D_3 on phospholipid-composition of rat renal brush-border membrane. Am J Physiol 249:F117–F123
22. Boyan BD, Dean DD, Sylvia VL, Schwartz Z 1994 Non-genomic regulation of extracellular matrix events by vitamin D metabolites. J Cell Biochem 56:331–339
23. Khare S, Tien X-Y, Wilson D, Wali RK, Bissonnette BM, Scaglione-Sewell B, Sitrin MD, Brasitus TA 1994 The role of protein kinase-Cα in the activation of particulate guanylate cyclase by 1α,25-dihydroxyvitamin D_3 in CaCo-2 cells. Endocrinology 135:277–283
24. Bissonnette M, Tien X-Y, Niedziela SM, Hartmann SC, Frawley Jr BP, Roy HK, Sitrin MD, Perlman RL, Brasitus TA 1994 1,25(OH)2 vitamin D_3 activates PKC-α in CaCo-2 cells: A mechanism to limit secosteroid-induced rise in [Ca^{2+}]i. Am J Physiol 267:G465–G475
25. Beno DWA, Brady LM, Bissonnette M, Davis BH 1995 Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D_3-stimulated Egr induction. J Biol Chem 270:3642–3647
26. Bissonnette M, Wali RK, Hartmann SC, Niedziela SM, Roy HK, Tien X-Y, Sitrin MD, Brasitus TA 1995 1,25-dihydroxyvitamin D_3 and 12-O-tetradecanoyl phorbol 13-acetate cause differential activation of Ca^{2+}-dependent and Ca^{2+}-independent isoforms of protein kinase C in rat colonocytes. J Clin Invest 95:2215–2221
27. Bhatia M, Kirkland JB, Meckling-Gill KA 1995 Monocytic differentiation of acute promyelocytic leukemia cells in response to 1,25-dihydroxyvitamin D_3 is independent of nuclear receptor binding. J Biol Chem 270:15962–15965
28. Slater SJ, Kelly MB, Taddeo FJ, Larkin JD, Yeager MD, McLane JA, Ho C, Stubbs CD 1995 Direct activation of protein kinase C by 1α,25-dihydroxyvitamin D_3. J Biol Chem 270:6639–6643
29. Norman AW, Mitra MN, Okamura WH, Wing RM 1975 Vitamin D: 3-Deoxy-1α-hydroxy vitamin D_3, a biologically active analog of 1α,25-dihydroxyvitamin D_3. Science 188:1013–1015
30. Wing RM, Okamura WH, Rego A, Pirio MR, Norman AW 1975 Studies on vitamin D and its analogs VII: Solution conformations of vitamin D_3 and 1,25-dihydroxyvitamin D_3 by high resolution proton magnetic resonance spectroscopy. J Am Chem Soc 97:4980–4985
31. Okamura WH, Midland MM, Hammond MW, Rahman NA, Dormanen MC, Nemere I, Norman AW 1994 Conformation and related topological features of vitamin D: structural relationships. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D, a Pluripotent Steroid

Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications. Walter de Gruyter, Berlin, pp 12–20
32. Wing RM, Okamura WH, Pirio MR, Sine SM, Norman AW 1974 Vitamin D_3: conformations of vitamin D_3, 1α,25-dihydroxyvitamin D_3, and dihydrotachysterol$_3$. Science 186:939–941
33. Okamura WH, Midland MM, Hammond MW, Rahman NA, Dormanen MC, Nemere I, Norman AW 1995 Chemistry and conformation of vitamin D molecules. J Steroid Biochem Mol Biol 53:603–613
34. Norman AW, Okamura WH, Farach-Carson MC, Allewaert K, Branisteanu D, Nemere I, Muralidharan KR, Bouillon R 1993 Structure-function studies of 1,25-dihydroxyvitamin D_3 and the vitamin D endocrine system. 1,25-dihydroxy-pentadeuterio-previtamin D_3 (as a 6-s-cis analog) stimulates nongenomic but not genomic biological responses. J Biol Chem 268:13811–13819
35. Dormanen MC, Bishop JE, Hammond MW, Okamura WH, Nemere I, Norman AW 1994 Nonnuclear effects of the steroid hormone $1\alpha,25(OH)_2$-vitamin D_3: analogs are able to functionally differentiate between nuclear and membrane receptors. Biochem Biophys Res Commun 201:394–401
36. Bishop JE, Collins ED, Okamura WH, Norman AW 1994 Profile of ligand specificity of the vitamin D binding protein for 1α,25(OH)2-vitamin D_3 and its analogs. J Bone Miner Res 9:1277–1288
37. Wecksler WR, Norman AW 1980 Structural aspects of the binding of 1α,25-dihydroxyvitamin D_3 to its receptor system in chick intestine. In: Methods in Enzymology: Vitamins and Co-Enzymes. Academic Press, New York, vol 67:494–500
38. Norman AW 1965 Actinomycin D and the response to vitamin D. Science 149:184–186
39. Farach-Carson MC, Abe J, Nishii Y, Khoury R, Wright GC, Norman AW 1993 22-Oxacalcitriol: Dissection of $1,25(OH)_2$ D_3 receptor-mediated and Ca^{2+} entry-stimulating pathways. Am J Physiol 265:F705–F711
40. Mangelsdorf DJ, Koeffler HP, Donaldson CA, Pike JW, Haussler MR 1984 1,25-Dihydroxyvitamin D_3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. J Cell Biol 98:391–398
41. Collins ED, Jones GI, Uskokovic MR, Koeffler HP, Norman AW 1988 $1\alpha,25(OH)_2$ vitamin D_3 analogs: comparison of $1\alpha,25(OH)_2D_3$ receptor ligand specificity and biological activity in the chick and human promyelocytic (HL-60) cells. In: Norman AW, Schaefer K, Grigoleit HG, Herrath Dv (eds) Vitamin D: Molecular, Cellular and Clinical Endocrinology. Walter de Gruyter, Berlin, pp 433–434
42. Cho YL, Christensen C, Saunders DE, Lawrence WD, Deppe G, Malviya VK, Malone JM 1991 Combined effects of 1,25-dihydroxyvitamin D_3 and platinum drugs on the growth of MCF-7 cells. Cancer Res 51:2848–2853
43. Brenner RV, Shabahang M, Schumaker LM, Nauta RJ, Uskokovic MR, Evans SRT, Buras RR 1995 The antiproliferative effect of vitamin D analogs on MCF-7 human breast cancer cells. Cancer Lett 92:77–82
44. Norman AW 1968 The mode of action of vitamin D. Biol Rev 43:97–137
45. Khoury RS, Weber J, Farach-Carson MC 1995 Vitamin D metabolites modulate osteoblast activity by Ca^{2+} influx-independent genomic and Ca^{2+} influx-dependent nongenomic pathways. J Nutr 125[Suppl]:1699S–1703S
46. de Boland AR, Norman AW 1997 $1\alpha,25(OH)_2$-vitamin D_3 signaling in chick enterocytes: enhancement of tyrosine phosphorylation and rapid activation of MAP-kinase. Mol Endocrinol, submitted
47. Song X, Norman AW 1997 Activation of $p42^{mapk}$ by $1\alpha,25(OH)_2$-vitamin D_3 6-s-*cis* and 6-s-*trans* locked analogs in human NB4 promyelocytic leukemia cells. Endocrinology, submitted
48. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL 1993 A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315–319
49. Nemere I, Dormanen MC, Hammond MW, Okamura WH, Norman AW 1994 Identification of a specific binding protein for 1α,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 269:23750–23756
50. Zhou L-X, Norman AW 1995 $1\alpha,25(OH)_2$-vitamin D_3 analog structure-function assessment of intestinal nuclear receptor occupancy with induction of calbindin-D28K. Endocrinology 136:1145–1152
51. Norman AW, Nemere I, Raman Muralidharan K, Okamura WH 1992 $1\alpha,25(OH)_2$-vitamin D_3 is an antagonist of $1\alpha,25(OH)_2$-vitamin D_3 stimulated transcaltachia (the rapid hormonal stimulation of intestinal calcium transport). Biochem Biophys Res Commun 189:1450–1456
52. Norman AW, Bouillon R, Farach-Carson MC, Bishop JE, Zhou L-X, Nemere I, Zhao J, Muralidharan KR, Okamura WH 1993 Demonstration that 1α,25-dihydroxyvitamin D_3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of 1α,25-dihydroxyvitamin D_3. J Biol Chem 268:20022–20030
53. Bouillon R, Van Baelen H 1981 Transport of vitamin D: significance of free and total concentrations of the vitamin D metabolites. Calcif Tissue Int 33:451–453
54. VanBaelen H, Allewaert K, Bouillon R 1988 New aspects of the plasma carrier protein for 25-hydroxycholecalciferol in vertebrates. Ann NY Acad Sci 538:60–68
55. Muralidharan KR, deLera AR, Isaeff SD, Norman AW, Okamura WH 1993 Studies on the A-ring diastereomers of 1α,25-dihydroxyvitamin D_3. J Org Chem 58: 1895–1899
56. VanAlstyne EM, Norman AW, Okamura WH 1994 7,8-cis geometric isomers of the steroid hormone 1α,25-dihydroxyvitamin D_3. J Am Chem Soc 116:6207–6216
57. Wecksler WR, Norman AW 1980 Structural aspects of the binding of 1α,25-dihydroxyvitamin D_3 to its receptor system in chick intestine. Methods Enzymol 67:494–500
58. Ridall AL, Daane EL, Dickinson DP, Butler WT 1995 Characterization of the rat osteopontin gene: evidence for two vitamin D response elements. Ann NY Acad Sci 760:59–66