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NONNUCLEAR EFFECTS OF THE STEROID HORMONE 1α,25(OH)$_2$-VITAMIN D$_3$: ANALOGS ARE ABLE TO FUNCTIONALLY DIFFERENTIATE BETWEEN NUCLEAR AND MEMBRANE RECEPTORS

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The steroid hormone 1α,25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$] stimulates biological responses via both genomic mechanisms and nongenomic mechanisms (opening of voltage-gated Ca^{2+} channels). We report here that the two closed B-ring steroid analogs of 1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$-7-dehydrocholesterol and 1α,25(OH)$_2$-lumisterol$_3$, are able to generate the nongenomic response, transcaltachia, without the ability to compete with the natural metabolite for binding to its nuclear receptor. We propose that the nongenomic membrane associated receptor can accept the ligand in its closed "6-s-*cis*" conformation whereas the nuclear receptor prefers the extended "6-s-*trans*" conformer.

Vitamin-D$_3$ is activated by a series of two obligatory, sequential hydroxylations in the liver and kidney which generate the hormonally active form 1α,25-dihydroxyvitamin D$_3$, [1α,25(OH)$_2$D$_3$] (1). 1α,25(OH)$_2$D$_3$ is unique as a steroid hormone in that it can exist in many different conformations by virtue of three key structural aspects of the compound; these include the 8 carbon side chain, the "broken" or *seco*-B-ring, and the A-ring (2). Accordingly, the molecule possesses a wide range of possible conformations with which its receptor(s) may selectively interact to initiate the generation of its many known biological responses. These responses can now be separated into two distinct classes based on the transduction mechanism utilized by the hormone: the first being the classical pathway of gene transcription regulation which involves the relatively well characterized nuclear vitamin D receptor (N-VDR) system (3) and the second consisting of the rapid, nongenomic response system which probably involves a membrane associated vitamin D receptor (M-VDR) and the opening of Ca^{2+} channels (4,5). Best studied of this second mechanism is the rapid (within 2 min) stimulation by 1α,25(OH)$_2$D$_3$ of $^{45}Ca^{2+}$ transport from the lumen of isolated chick duodenum to the venous effluent which is termed *transcaltachia* (6). At issue here is whether the ligand binding domains of the two receptors (N-VDR & M-VDR) recognize the same shape of this conformationally mobile seco-steroid.

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Various analogs of 1α,25(OH)$_2$D$_3$ have been employed to help characterize both the nuclear and the transcaltachic responses (7,8). Previous studies have shown that the extended 6-s-*trans* conformer (see Fig.1) is the thermodynamically favored structure in solution (9,10). This is also thought to be the preferred form of the steroid for binding with its nuclear receptor. However, insight into the possibility of other receptor-ligand preferences was first indicated for transcaltachia when a pentadeuterio-1α,25(OH)$_2$-pre-D$_3$ analog (designated analog **HF**; Fig.1) with thermodynamic stability in the more steroid-like 6-s-*cis* conformation was found to be able to stimulate Ca^{2+} transport at a level equivalent to that of the natural hormone (11). In this communication, two closed B-ring steroid analogs were assayed for their ability to initiate transcaltachia as well as to compete with 1α,25(OH)$_2$D$_3$ for binding to both the N-VDR and the serum vitamin D transport protein (DBP). We report here that the closed B-ring analogs 1α,25(OH)$_2$-7-dehydrocholesterol (**JM**) and 1α,25(OH)$_2$-lumisterol$_3$ (**JN**) (Fig. 1), will both stimulate transcaltachia (**JN** > **JM**) while neither will compete with 1α,25(OH)$_2$D$_3$ for binding to either the N-VDR or DBP.

MATERIALS AND METHODS

Materials

$^{45}Ca^{2+}$ (1 Ci/mmol) was obtained from New England Nuclear (Dupont Chemical) Boston, MA. Analogs HF and HJ were each synthesized as previously reported (12,13). The synthesis of JM and JN are described below. 1α,25(OH)$_2$D$_3$ (analog **C**) was a kind gift from Dr. Milan Uskokovic, Hoffmann LaRoche, Nutley, NJ. Human DBP (sold as Gc-globulin) was obtained from Sigma Chemical, St. Louis, MO.

Synthesis of **JM** [1α,25-(OH)$_2$-7-Dehydrocholesterol] and **JN** [1α,25-(OH)$_2$-7-Lumisterol$_3$]. A solution of the known (12) 1α,25-(OH)$_2$-previtamin D$_3$ (120 mg) in methanol was irradiated (Hanovia 450 watt medium pressure mercury lamp, pyrex filter, λ > 300 nm) for 3 h at room temperature. The solution was concentrated and subjected to HPLC (Rainin Microsorb, 5 μm silica, 10 mm x 25 cm, 11% isopropanol/hexanes) to afford in order of elution **JM** (9.1 mg, 7.6%), **JN** (15.0 mg, 12.5%) and starting 1α,25-(OH)$_2$-previtamin D$_3$ (10.6 mg, 8.8%). **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~5.6 Hz), 1.22 (6H, $C_{26,27}$-CH_3, s), 2.35 (1H, apparent t, J~12.7 Hz), 2.55 (1H, d with fine structure, J~14.2 Hz), 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~5.5 Hz, 2.8 Hz, 2.8 Hz), 5.73 (1H, H_7 or 6, dd, J~5.5 Hz, 2.2 Hz). ^{13}C-NMR ($CDCl_3$): δ 11.9, 16.3, 18.8, 20.8, 20.9, 23.0, 28.1, 29.2, 29.4, 36.1, 36.4, 38.0, 38.5, 39.2, 40.0, 43.1, 44.4, 54.7, 55.8, 65.5, 71.1, 73.0, 115.2, 122.1, 141.4. 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); λ_{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). MS: (CI, CH_4) m/z 417 (28, MH), 400 (67), 381 (31), 354 (11), 338 (6), 323 (6), 297 (4), 267 (4), 251 (8), 225 (10), 211 (10), 197 (11), 171 (19), 157 (15), 119 (12), 105 (15), 91 (14), 81ε (14), 69 (27), 59 (base). **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~5.2 Hz), 1.21 (6H, $C_{26,27}$-CH_3, s), 2.50 (2H, m), 4.10 (1H, H_1, dd, J~9.2 Hz, 4.8 Hz), 4.14 (1H, H_3, dd, J~3.0 Hz, 3.0 Hz), 5.45 (1H, H_6 or 7, m), 5.75 (1H, H_7 or 6, dd, J~5.1 Hz, 1.7 Hz). ^{13}C-NMR ($CDCl_3$): δ 7.4, 18.3, 18.5, 20.9, 21.7, 22.6, 28.8, 29.2, 29.4, 29.7, 36.2, 37.5, 38.9, 39.5, 41.4, 43.9, 44.4, 46.2, 49.5, 57.3, 66.2, 71.1, 75.8, 115.5, 123.6, 137.2, 142.2. 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 (calcd. for $C_{27}H_{44}O_3$ plus H, 417.3370). MS (CI, CH_4): m/z 417 (86, MH), 400 (base), 382 (60), 366 (13), 343 (8), 325 (6), 311 (5), 287 (15), 269 (13), 251 (9), 227 (13), 213 (9), 174 (46), 157 (21), 143 (14), 119 (7), 105 (8), 95 (8), 81 (8), 69 (14), 59 (38).

Animals

White Leghorn cockerels (Hyline International, Lakeview, CA) were obtained on the day of hatch and maintained on a vitamin D supplemented diet (O.H. Kruse Grain &

Milling, Ontario, CA) for 4-5 weeks (400-600 gm). When vitamin D deficient chicks were employed, they were raised for 4 weeks on a rachitogenic diet (14).

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

Measurement of Ca^{2+} transport (transcaltachia) was carried out in perfused chick duodena essentially as previously described (6,15). Briefly, chicks were anesthetized with chloropent (0.3ml/100gm) and the duodenal loop exposed. Three pairs of blood vessels branching off from the celiac artery were ligated prior to cannulation of the celiac artery itself. The arterial perfusion was initiated with Gey's Balanced Salt Solution (GBSS) modified to contain 0.9 mM $CaCl_2$ and oxygenated with 95% O_2/5% CO_2, at a flow rate of 2 ml/min. An auxiliary pump was used for the introduction of vehicle (0.005% ethanol v/v, final concentration) or test analogs plus albumin (0.125% w/v, final concentration) to the vascular perfusate at a rate of 0.25 ml/min. The intestinal loop was then excised and the lumen flushed and filled with GBSS (lacking $NaHCO_3$ and glucose) containing $^{45}Ca^{2+}$ (5 μCi/ml). The lumenal solution is renewed constantly at a rate of 0.25 ml/min to insure a steady concentration of $^{45}Ca^{2+}$ at the brush border of the epithelia. The intestinal preparation at 27°C was kept moist under layers of saline-dampened cheese cloth. Each duodenum was perfused with control medium (vehicle) for 20 min after filling the lumen with $^{45}Ca^{2+}$ to establish the basal transport rate. The tissue was then either exposed to the test analog or continued on vehicle for an additional 40 min. The venous effluent was collected at 2 min intervals during basal and treatment periods and assayed for $^{45}Ca^{2+}$ activity via liquid scintillation. The results are expressed as the ratio of the $^{45}Ca^{2+}$ appearing at each interval of the treated phase (40 min = 20 data points) over the average basal rate (initial 20 min) (6,11).

Receptor and DBP Binding

The relative ability of an analog to compete with [^{3}H]-1α,25(OH)$_2$D$_3$ for binding to the chick intestinal nuclear receptor was carried out *in vitro* according to our standard procedures (16). In this assay, increasing concentrations of nonradioactive 1α,25(OH)$_2$D$_3$ or the test analog are incubated with a fixed saturating amount of [^{3}H]-1α,25(OH)$_2$D$_3$ and chick intestinal nuclear extract obtained from vitamin D-deficient chicks; the reciprocal of the percentage of maximal binding of [^{3}H]-1α,25(OH)$_2$D$_3$ was then calculated and plotted as a function of the relative concentration of the analog and [^{3}H]-1α,25(OH)$_2$D$_3$. The competitive index value for each analog is then normalized to a standard curve obtained with nonradioactive 1α,25(OH)$_2$D$_3$ as the competitive steroid and placed on a linear scale of Relative Competitive Index(s) (RCI), where the RCI of 1α,25(OH)$_2$D$_3$ is by definition 100.

The relative ability of vitamin D analogs to bind to the plasma transport protein, the vitamin D binding protein (DBP) were carried out in a similar fashion (17).

RESULTS AND DISCUSSION

The structure of the natural hormone, 1α,25(OH)$_2$D$_3$, in both its 6-s-*trans* and 6-s-*cis* conformations, as well as the structures of the other analogs are presented in Figure 1. Pre-vitamin D$_3$ is formed in the skin when the 9-10 carbon-carbon bond of 7-dehydrocholesterol is broken by UV radiation from sunlight. This "broken" or *seco*-B-ring allows a 360° range of rotational freedom about the 6-7 single bond and thus generates the *cis* and *trans* conformers. The A-ring also acquires a new dimension of internal mobility since it is no longer rigidly fused to the B-ring. The cyclohexane-like A-ring can rapidly interchange between a pair of chair-chair conformers effectively equilibrating the critical 1α- and 3ß-hydroxyl groups between equatorial and axial orientations (18). This observation guided the synthesis of our two closed B-ring analogs, **JM** and **JN**, which differ significantly only in the now fixed orientations of the two A-ring hydroxyls; **JM** is 1α-axial and 3ß-equatorial, **JN** is 1α-equatorial and 3ß-axial. Also it is apparent that **JM** and **JN** are

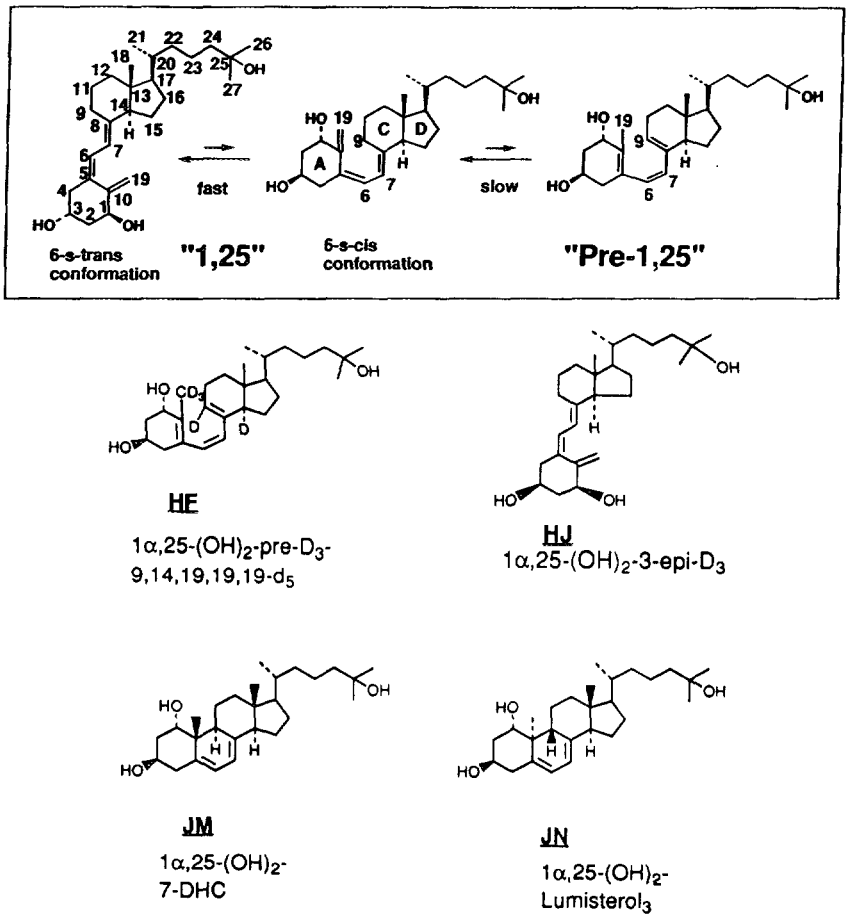

Fig. 1. Conformational changes of 1α,25(OH)$_2$D$_3$ about the 6-7 carbon bond and comparison to selected analogs. The seco-steroid hormone, 1α,25(OH)$_2$D$_3$ (structure C), can rotate 360^0 about the single bond between carbon six and carbon seven to assume either an extended 6-s-*trans* form (left structure) or steroid-like 6-s-*cis* form (middle structure). The pre-vitamin form is also shown (right structure). Analog **HJ**, 1α,25(OH)$_2$-epi-3-D$_3$, is a diastereomer of the natural metabolite which has its 3-hydroxyl in the "α" position instead of the normal "ß". This compound posesses a greater affinity for the DBP than 1α,25(OH)$_2$D$_3$ but loses much of its ability to bind to the N-VDR. Analogs of the 6-s-*cis* conformation that have been evaluated for activity include 1α,25(OH)$_2$-9,14,19,19,19-penatdeuterio-previtamin D$_3$ (**HF**), 1α,25-(OH)$_2$-7-dehydrocholesterol (**JM**), and 1α,25-(OH)$_2$-lumisterol$_3$ (**JN**).

analogs of the 6-s-*cis* form of 1α,25(OH)$_2$D$_3$; they can never exist in the extended 6-s-*trans* conformation.

Transcaltachia

Figures 2a and 2b illustrate the appearance of $^{45}Ca^{2+}$ in the venous effluent mediated by the two different concentrations of our test analogs, **JM** and **JN**, (Figs. 2a & 2b respectively), vehicle control (ethanol only) and 650pM 1α,25(OH)$_2$D$_3$ as positive control. The efficacy of 300pM **JM** in initiating transcaltachia is not significantly greater than control, and the response elicited at 650pM **JM** is only 60% of that induced by the

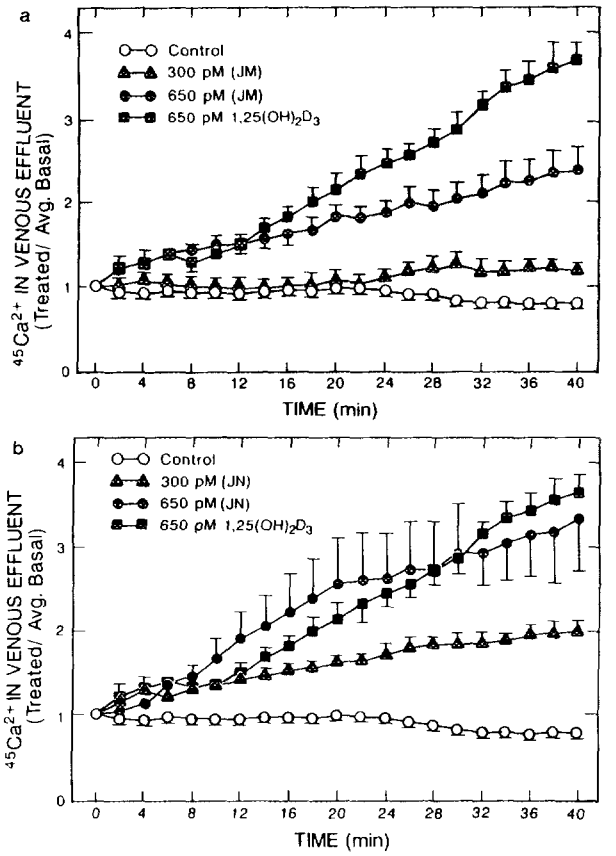

Fig. 2. Stimulated $^{45}Ca^{2+}$ transport in duodenal loops vascularly perfused with 1α,25(OH)$_2$D$_3$, or 1α,25(OH)$_2$-7-dehydrocholesterol (JM), or 1α,25(OH)$_2$-lumisterol$_3$ (JN). Duodenal loops from normal, vitamin D-replete chicks were lumenally perfused with $^{45}Ca^{2+}$ (5 μCi/ml of buffer) and vascularly perfused with control medium for the first 20 min, with collection of the venous effluent occurring at 2-min intervals during the final 10 min to establish basal transport rates. The duodena were then either re-exposed to control medium containing the vehicle ethanol (0.005%, final concentration) through the celiac artery, or vascularly perfused with 300 pM agonist, or 650 pM agonist. The venous effluent was again collected at 2 min intervals for liquid scintillation spectrophotometry. The results during the treated phase were normalized to the average basal transport for each duodenum. Values represent mean $\pm$ SEM for n = 4 in each group. (a) perfusion with JM; (b) perfusion with JN. Included in each graph are both vehicle control and 650pM 1α,25(OH)$_2$D$_3$ as positive control.

natural metabolite. Perfusion with JN, however, produced a stimulation nearly identical to that of 1α,25(OH)$_2$D$_3$ with JN achieving only a slightly lower ratio of transport of $^{45}Ca^{2+}$ than that achieved by 1α,25(OH)$_2$D$_3$.

Figures 3a and 3b present the dose response curves for both of the analogs. Each bar represents the 40 min data point of Figure 3 which is taken as the maximum response elicited by the analog at that concentration. Analog JN (Fig. 3a) eventually reaches the 4-fold plateau at 1300 pM which is the equivalent of the maximum stimulation achieved by 1α,25(OH)$_2$D$_3$ at 650 pM. The $^{45}Ca^{2+}$ transport ratio for analog JM at 650 pM (Fig. 3b) peaked at 2.5 and was not further increased as a consequence of increasing the JM concentration to 1300 pM.

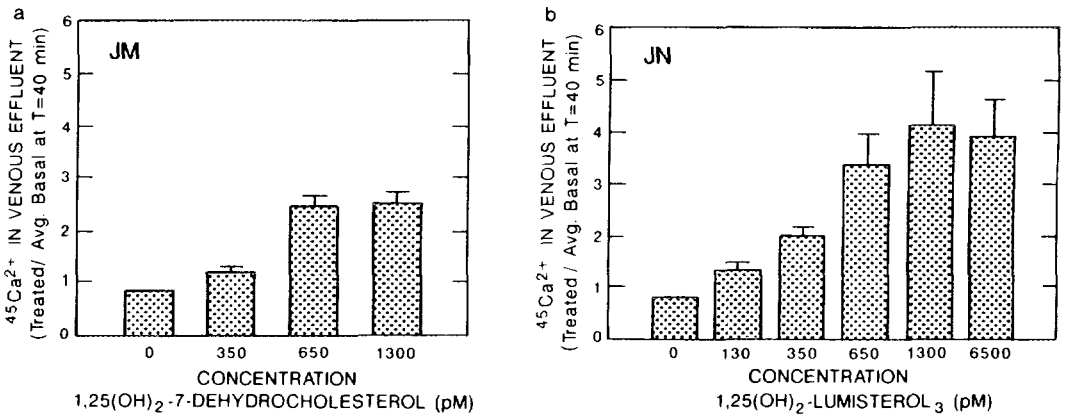

Fig. 3. Dose-response analyses of JM and JN as agonists for transcaltachia. Duodena were perfused as described in Fig. 2 with vehicle or a range of analog concentrations. Normalized transport after 40 min of perfusion is depicted for the indicated concentrations of (a) JM; (b) JN.

N-VDR & DBP Binding

As expected, neither of our closed "6-s-*cis*" analogs could significantly compete with 1α,25(OH)$_2$D$_3$ for binding to the nuclear receptor (Table 1, N-VDR-RCIs for JM and JN). Other studies have correlated very poor N-VDR binding to lack of genomic activity (7,8,11), so both of these analogs are also predicted to be ineffective gene regulators. Poor binding to the human serum DBP was expected as well.

The major circulating vitamin D metabolite in the serum is 25(OH)D$_3$ and it follows that this metabolite should have the highest affinity for the serum transport protein DBP, which is indeed the case (Table 1). However, binding to the nuclear receptor appears to require 1α-hydroxylation which also is known to reduce DBP binding significantly (19). Some 1α-hydroxy analogs such as analog HJ, 1α,25(OH)$_2$-3-epi-D$_3$, display intermediate

TABLE 1

RELATIVE COMPETITIVE INDEX (RCI)
FOR ANALOGS OF 1α,25(OH)$_2$D$_3$

Analog	RCI DBP	RCI N-VDR
1α,25(OH)$_2$D$_3$	100	100
25(OH)D$_3$	66,800	0.15 $\pm$ 0.05
HJ	800	24 $\pm$ 4.5
HF	8.6 $\pm$ 0.1	10 $\pm$ 6
JM	< 0.1	0.12 $\pm$ 0.05
JN	< 0.1	1.8 $\pm$ 0.5

The RCI values were determined as described under the Methods section. The RCI values for 1α,25(OH)$_2$D$_3$ are by definition set to 100%. DBP = human vitamin D binding protein; N-VDR = nuclear vitamin D receptor from the chick intestine.

DBP binding. Since both of our test analogs are 1α-hydroxy, we expected a reduced but still appreciable DBP binding unless the closed 6-s-*cis* conformation precluded any binding whatsoever. The DBP-RCI values in Table 1 indicate that competition for the DBP by both **JM** and **JN** was nonexistent.

CONCLUSIONS

The performance of 1α,25(OH)$_2$-lumisterol$_3$, analog **JN**, in our perfusion assay for transcaltachia is strong evidence that the membrane receptor for 1α,25(OH)$_2$D$_3$ can accommodate the ligand in its 6-s-*cis* rotational conformation. The poorer performance of 1α,25(OH)$_2$-7-dehydrocholesterol, analog **JM** with its 1α-hydroxyl locked axially instead of equatorially like **JN**, indicates that the requirements of this receptor are even more stringent than previously suspected. Finally, the inability of either **JN** or **JM** to compete with 1α,25(OH)$_2$D$_3$ for binding to its nuclear receptor or to DBP suggests that both of these proteins prefer the hormone in the extended 6-s-*trans* conformation.

Such dramatic differences in mode of action (nuclear vs. Ca^{2+} channel opening) and in receptor ligand conformational requirements strongly suggest the existence of not only two distinct transductional mechanisms but also two completely separate classes of receptor for the same ligand. Significant progress towards purification and characterization of the membrane associated receptor has already been accomplished in our laboratory (20).

1α,25(OH)$_2$D$_3$ has proven to be a very eclectic hormone with effects ranging from Ca^{2+} regulation to cellular differentiation. Much of the future promise of this research is based on the potential for identifying analogs that are specific for certain aspects of vitamin D function without affecting other responses such as bone Ca^{2+} mobilization and hypercalcemia. 1α,25(OH)$_2$D$_3$ has already been shown *in vitro* to inhibit cell proliferation and promote differentiation in cultures of acute myelogenous leukemia, (21) psoriasis, (22,23) and breast cancer cells (24,25). Hence, a thorough understanding of the structure-function relationships of this very unique hormone could reap valuable insights for future advances.

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