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STUDIES ON THE MODE OF ACTION OF CALCIFEROL XIX. A 24R-HYDROXYL-GROUP CAN REPLACE THE 25-HYDROXYL-GROUP OF 1α,25-DIHYDROXYVITAMIN D_3 FOR OPTIMAL BINDING TO THE CHICK INTESTINAL RECEPTOR†

Paul D. Siebert, Norio Ohnuma# and Anthony W. Norman+

Department of Biochemistry
University of California
Riverside, California 92521

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SUMMARY:

Various 1α-hydroxylated side chain analogs of vitamin D_3 have been studied for their ability to compete with 1α,25-dihydroxy[^{3}H]vitamin D_3 for binding to the chick intestinal receptor. Of the analogs examined, 1α,24R-dihydroxyvitamin D_3 was found to be nearly equivalent to 1α,25-dihydroxyvitamin D_3 in its ability to compete for receptor binding. However, this near equivalence was not shared by its stereoisomer, 1α,24S-dihydroxyvitamin D_3, which was only 10% as effective a competitor. It is proposed that the ability of a 24R-hydroxyl group to mimic the 25-hydroxyl group is not due to a lack of side chain specificity on the part of the receptor, but is instead due to the similar orientation of the 25-hydroxyl and the 24R-hydroxyl such that they can be accommodated equivalently by the receptor.

Introduction

The biological responses of vitamin D, mediated via 1α,25(OH)$_2$D$_3$,[1] are proposed to occur as a consequence of the formation of specific complexes with cytosol-receptor/binding proteins present in target tissues (2). This steroid-receptor complex then migrates to the nucleus and initiates the synthesis of specific mRNAs, including one for a vitamin D-dependent calcium

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Teijin Institute for Bio-Medical Research 3-9-2, Asahigaoka Hino-shi, Tokyo.

+ To whom all inquires should be made.

[1] Abbreviations employed for the various compounds discussed in this investigation are: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 24-OH-D_3, 24-hydroxyvitamin D_3; 24-OH-25-F-D_3, 24-hydroxy-25-fluorovitamin D_3; 1α,24(OH)$_2$D$_3$, 1α,24-dihydroxyvitamin D_3; 1α,24(OH)$_2$-25-F-D_3, 1α,24-dihydroxy-25-fluorovitamin D_3; 24,25(OH)$_2$D$_3$, 24,25-dihydroxyvitamin D_3; 1α,24,25(OH)$_3$D$_3$, 1α,24,25-trihydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1α-OH-$\Delta^{24,25}$-D_3, 1α-hydroxy-$\Delta^{24,25}$-vitamin D_3.

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binding protein (3). The fact that vitamin D_3 must be hydroxylated at both the 1α and 25-positions *in vivo* before it can exert its biological functions has led to the concept that the 1α and 25-hydroxyl groups are critical for the activity of this seco-steroid (4). However, synthetic analogs of vitamin D_3 which are hydroxylated at the 24-position, but not the 25 position, have also been shown to have considerable biological activity when assayed *in vivo* (5-8). Such analogs include; 24-hydroxyvitamin D_3 [24-OH-D_3] (5), 1α,24-dihydroxyvitamin D_3 [1α,24(OH)$_2D_3$] (6), 24-hydroxy-25-fluorovitamin D_3 [24-OH-25-F-D_3] (7) and 1α,24-dihydroxy-25-fluorovitamin D_3 [1α,24(OH)$_2$-25-F-D_3] (8). Since both 24-OH-D_3 and 24-OH-25-F-D_3 have no biological activity in nephrectomized animals, the activities of these compounds may be due to their metabolism in the kidney to the corresponding 1α-hydroxylated analogs (6,7). But for 24-OH-D_3 other interpretations are possible since this analog has been shown to undergo metabolism to 24,25(OH)$_2D_3$ and 1α,24,25-(OH)$_3D_3$ (9). The analogs 24-OH-25-F-D_3 and 1α,24(OH)$_2$-25-F-D_3 can induce bio-responses which were nearly identical to their corresponding naturally occurring vitamin D_3 metabolites 25-OH-D_3 (7) and 1α,25(OH)$_2D_3$ (8). Since these synthetic fluoro-compounds cannot undergo hydroxylation at the 25-position, their potency could be due to the ability of a 24-hydroxyl group to mimic a 25-hydroxyl *in vivo*.

This study was undertaken to compare the ability of various 1α-hydroxylated analogs to compete with 1α,25(OH)$_2$[^{3}H]D_3 for binding to the chick intestinal cytosol receptor system *in vitro*. This cytosol assay system (10), as well as the cytosol-chromatin system (11,12), have previously proven useful to define the very high degree of structural specificity of the intestinal receptor system for both the A-ring (4,10,11) as well as the side chain (12) of the ligand. The results presented here indicate that a 24-hydroxyl group located in the R, but not the S, configuration can effectively substitute for the 25-hydroxyl group in the 1α,25(OH)$_2D_3$ molecule. This finding is discussed in terms of the ligand specificity exhibited by the intestinal recep-

tor and the steering role played by the side chain portion of the vitamin D_3 molecule.

Material and Methods

Chemicals. $1\alpha,25(OH)_2$[26,27-methyl-^{3}H]vitamin D_3 (9 Ci/mmol) was produced enzymatically from 25-OH-[26,27-methyl-^{3}H]vitamin D_3 (purchased from Amersham-Searle, Chicago, IL) (13). Nonradioactive $1\alpha,25(OH)_2$vitamin D_3 and $1\alpha,24R,25(OH)_3$vitamin D_3 were kindly provided by Dr. M. Uskokovic of Hoffmann-LaRoche (Nutley, NJ). $1\alpha,24R(OH)_2$vitamin D_3, $1\alpha,24S(OH)_2$ vitamin D_3 and 1α-OH-$\Delta^{24,25}$-vitamin D_3 were gifts of Dr. K. Bannai of the Teijin Institute for Bio-Medical Research (Asahigaoka Hino-shi, Tokyo). 1α-OH-vitamin D_3 was kindly supplied by Dr. J. Babcock of the Upjohn Company (Kalamazoo, MI). All steroids were pure as evaluated by high pressure liquid chromatography and their UV absorption spectra. Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories (Richmond, CA).

Animals. One-day-old White Leghorn cockerels (obtained from Pace/Setter Hatchery, Ontario, CA) were raised on a vitamin D-deficient diet (13) for 4-5 weeks prior to sacrifice.

Cytosol preparation. All operations were carried out at 0-4°C. Upon sacrifice, duodenal loops were removed, opened longitudinally, rinsed with ice-cold saline (0.15 M NaCl) and the mucosa was scraped from the underlying serosa using a glass microscope slide. A 20% homogenate (w/v) of the mucosa was prepared in buffer containing 0.3 M KCl, 10 mM Tris/HCl, 1 mM EDTA and 0.5 mM DTT, pH 7.5 using a Potter-Elvehjem homogenizer equipped with a Teflon pestle (10-12 strokes). The homogenate was then centrifuged at 4300 x g for 10 min. The resulting supernatant was centrifuged at 105,000 x g for 60 min in a Beckman (model L2-65B) ultracentrifuge to obtain the cytosol fraction. The floating lipid layer was removed and the remaining cytosol was lyophilized and stored at -20°C.

Competitive binding assay. The competitive binding assay was carried out by a hydroxylapatite batch assay (10). An aliquot (1.3-1.7 pmol) of $1\alpha,25(OH)_2$-[26,27-methyl-^{3}H]D_3 with a specific activity of 9 Ci/mmol and the appropriate amounts of the analog were pipetted into 1.8 ml polypropylene tubes (Sarstedt) in a small volume of ethanol. After drying down under nitrogen, the steroids were redissolved in 0.02 ml of ethanol with gentle vortexing. To initiate the binding reaction, 0.2 ml of the intestinal cytosol was added to each tube. Following incubation for 16 hours at 4°C, the tubes were placed into an ice-water bath and 0.4 ml hydroxylapatite slurry (50% v/v in 0.01 M K_2HPO_4, 0.1 M KCl, pH 7.5) were added. After frequent gentle vortexing for 15 min the samples were centrifuged in a Beckman Microfuge B for 2 sec. The hydroxylapatite pellets were washed three times with 0.8 ml of 10 mM Tris/HCl - 0.5% Triton X-100, pH 7.5 by vortexing and subsequent centrifugation as above. The washed pellets were extracted twice with 0.8 ml of methanol:chloroform (2:1) and the radioactivity in the extracts was determined in 8 ml of liquid scintillation cocktail (butyl-PBD; 5.0 g of 2-[4'-tert-butylphenyl-5-(4'-biphenyl-3,4-oxadiazole)] per liter of toluene) by liquid scintillation spectrophotometry. To correct for nonspecific binding, the amount of $1\alpha,25(OH)_2$[^{3}H]D_3 bound when incubated with a 150 fold excess of nonradioactive $1\alpha,25(OH)_2D_3$ was subtracted from the total amounts of $1\alpha,25$-$(OH)_2$[^{3}H]D_3 bound when incubated with increasing concentrations of the analog.

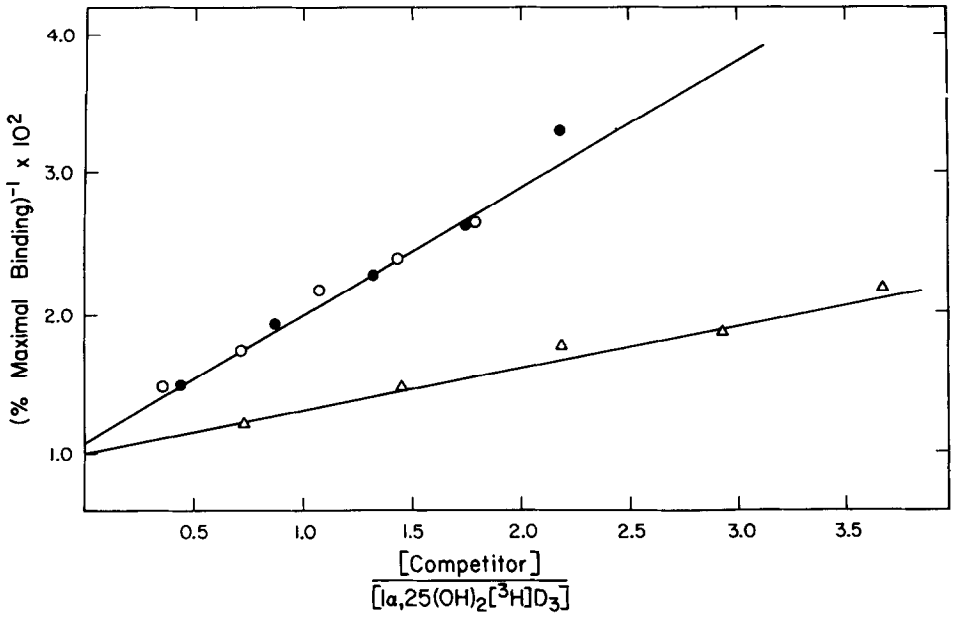
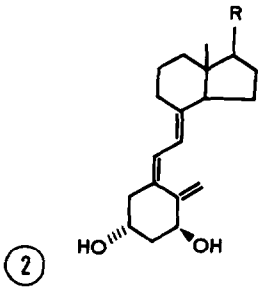


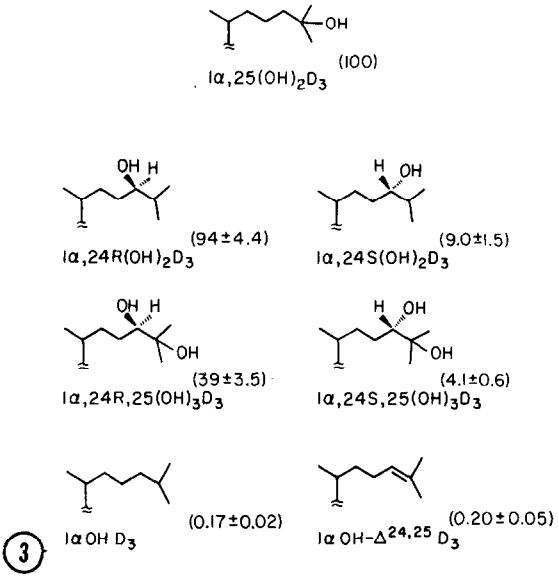
Fig. 1. Steroid competition analysis of side chain analogs of 1α,25(OH)$_2$D$_3$. Increasing concentrations of 1α,24R(OH)$_2$D$_3$ (O); 1α,24R,25(OH)$_3$D$_3$ (Δ) or 1α,25(OH)$_2$D$_3$ (●) were incubated for 16 hrs at 4°C with chick intestinal cytosol in the presence of a saturating concentration of 1α,25(OH)$_2$[^{3}H]D$_3$. The reciprocal of the percentage of maximal binding is plotted as a function of the relative concentrations of the nonradioactive steroid and 1α,25(OH)$_2$-[^{3}H]D$_3$. Each point represents the average of duplicate determinations.

Results

The ability of each analog to compete with 1α,25(OH)$_2$[^{3}H]D$_3$ for binding to the chick intestinal receptor was quantitated by measuring the decrease in specific binding of 1α,25(OH)$_2$[^{3}H]D$_3$ when increasing concentrations of each analog were incubated with a fixed saturating concentration of 1α,25(OH)$_2$-[^{3}H]D$_3$. The reciprocal of the percentage of maximal binding of 1α,25(OH)$_2$-[^{3}H]D$_3$ was then calculated and plotted as a function of the relative concentrations of the analog and 1α,25(OH)$_2$[^{3}H]D$_3$. As shown in Figure 1, such plots give linear curves characteristic for each analog, the slopes of which are equal to the analogs competitive index value α (14). The competitive index value for each analog is then normalized to a standard curve obtained using nonradioactive 1α,25(OH)$_2$D$_3$ as the competing steroid and placed on a linear scale of relative competitive index (RCI) using the relationship:



(2)



(3)

Fig. 2. The structure of 1α-OH-D_3. R represents the side chain.

Fig. 3. The side chain structures of the 1α,25(OH)$_2$$D_3$ molecule and the 1α-hydroxylated side chain analogs examined, indicating their respective RCI values. RCI values were calculated as described under Results. RCI values are expressed as the mean of triplicate determinations ± SEM.

$$RCI = \frac{\alpha_{analog}}{\alpha_{1\alpha,25(OH)_2D_3}} \times 100$$

On this scale 1α,25(OH)$_2$$D_3$ is defined as 100.

Shown in Figure 2 is the structure of 1α-OH-D_3, the parent compound for 1α,25(OH)$_2$$D_3$ and the analogs examined. Figure 3 shows the side chain portions of the 1α,25(OH)$_2$$D_3$ molecule and the various side chain analogs indicating their respective RCI values. Of all the analogs examined 1α,24R(OH)$_2$$D_3$ was found to be the best competitor being nearly equivalent to 1α,25(OH)$_2$$D_3$ in its ability to compete for receptor binding. Its epimer, 1α,24S(OH)$_2$$D_3$, which differs only in the configuration of the 24-hydroxyl was only 10% as effective a competitor. The trihydroxylated analogs 1α,24R,25(OH)$_3$$D_3$ and 1α,24S,25(OH)$_3$$D_3$ were also examined. 1α,24R,25(OH)$_3$$D_3$ was found to be 39% as effective a competitor as 1α,25(OH)$_2$$D_3$ and its epimer, 1α,24S,25(OH)$_3$$D_3$, 4% as effective. 1α-OH-D_3 and 1α-OH-$\Delta^{24,25}$-D_3, which lack hydroxyl groups

on the side chain, were found to be relatively poor competitors, both having about 0.2% of the activity of $1\alpha,25(OH)_2D_3$.

Discussion

Since both the R and S-epimers of $1\alpha,24(OH)_2D_3$ were found to be much better competitors than 1α-OH-D_3 and 1α-OH-$\Delta^{24,25}$-D_3, it can be concluded that a hydroxyl group located at the 24-position can readily satisfy the requirement for side chain hydroxylation. Further, the ability of $1\alpha,24R$-$(OH)_2D_3$ to compete nearly as effectively as $1\alpha,25(OH)_2D_3$ indicates that the hydroxyl group located at the 24-position in the R configuration can substitute virtually equivalently for a 25-hydroxyl group. This suggests that the high biological potency of 24R-hydroxylated analogs of vitamin D_3 is due to the near equivalence of $1\alpha,24R(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ rather than to the production of the trihydroxymetabolite, $1\alpha,24R,25(OH)_3D_3$, as had been previously proposed (5). As in prior studies (15,16) the addition of a hydroxyl group at the 24-position to $1\alpha,25(OH)_2D_3$ results in an apparent reduction in the ability of the 25-hydroxyl group to form a stabilizing interaction with the receptor. Although the reason for this apparent reduction is not clear, it is certainly consistent with the reduced biological activity of $1\alpha,24R,25(OH)_3D_3$ *in vivo* (17).

A unique property of the vitamin D_3 steroid is the conformational mobility of the A-ring. This property poses some interesting questions concerning the way in which the steroid and receptor molecules associate. It has been proposed that the interaction of the side chain portion of the $1\alpha,25(OH)_2D_3$ molecule with the receptor allows for the capture of the conformationally active form of the A-ring (4). It would follow that analogs having slightly altered side chains should be greatly reduced in their ability to compete for receptor binding. The ability of $1\alpha,24R(OH)_2D_3$ to compete equally well with $1\alpha,25(OH)_2D_3$ does not argue against this hypothesis since molecular model building indicates that the 24R- and 25-hydroxyl groups, the side chain methylene groups and the terminal dimethyl groups can

all be oriented in a nearly equivalent manner without introducing eclipsing interactions. Indeed, the receptor stereoselectivity for the 24R-hydroxyl and the reduction in binding capability when hydroxyl groups are present at both the 24 and 25 positions is certainly consistent with this hypothesis. Thus, it is proposed that the ability of $1\alpha,24R(OH)_2D_3$ to compete as effectively as $1\alpha,25(OH)_2D_3$ for binding is not due to the lack of receptor specificity for the side chain but due to the near equivalence of these ligands in the receptor binding site.

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