Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 496-501

24R,25-DIHYDROXYVITAMIN D_3 REGULATES 1,25-DIHYDROXYVITAMIN D_3 BINDING TO ITS CHICK INTESTINAL RECEPTOR

Francois Wilhelm and Anthony W. Norman+

Department of Biochemistry, University of California, Riverside, CA 92521

Received November 30, 1984

We have studied the binding of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] to its crude chromatin chick intestinal receptor in the absence or presence of a ten-fold excess of 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$] for each concentration of [^{3}H]-1,25$(OH)_2D_3$ studied. We have found a significant shift to the right in the binding of 1,25$(OH)_2D_3$ to its receptor in the presence of this excess of 24R,25$(OH)_2D_3$. As a result, the affinity was found to be significantly reduced, the apparent dissociation constants varied from 0.97±0.09 (n = 5) to 1.36±0.04 nM ($p < 0.01$). This reduction was related to a significant decrease in the positive cooperativity for the apparent Hill coefficient from n_H = 1.49±0.06 to n_H = 1.26±0.06 ($p < 0.03$) in the binding of 1,25$(OH)_2D_3$ to its receptor. There was no significant change in the capacity of the receptor (189±11 compared to 200±9 fmoles/mg protein). These results suggest that the intestinal 1,25$(OH)_2D_3$ receptor must also have a binding recognition site for 24R,25$(OH)_2D_3$ which is postulated to play a regulatory role in the 1,25$(OH)_2D_3$ receptor's ligand binding properties.

It is generally accepted that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] action in the target intestinal mucosa is mediated through its acting analogously to that of a classical steroid hormone, by an interaction with a specific high affinity receptor binding protein (1). We have recently reported (2) the existence of a positive cooperativity mechanism in the binding of 1,25$(OH)_2D_3$ to the two sites of its chick intestinal receptor. Additionally, this cooperativity was found to play a key role in the regulation of the receptor's affinity, in response to varying demands of dietary calcium and phosphorus (3).

A second dihydroxylated vitamin D metabolite, also produced by the kidney is 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$]. 24R,25$(OH)_2D_3$ production is stimulated at the kidney level by 1,25$(OH)_2D_3$, probably by an induction of

+ To whom all inquiries should be addressed.

0006-291X/85 $1.50

the $25(OH)D_3$-24-hydroxylase (4); under normal physiological circumstances both $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ are co-produced and distributed systemically via the blood transport protein, D-binding protein. The biological role of $24R,25(OH)_2D_3$ is still controversial, although some evidence for an inhibition in vivo of PTH secretion (5), as well as a stimulation of DNA synthesis in chondrocytes (6) have recently been reported. Synergistic effects of $24R,25(OH)_2D_3$ on $1,25(OH)_2D_3$ action have also been described on the egg hatchability (7) and on the maturation of chondrocytes (8). However, to date, there is no evidence of a direct interaction of $24R,25(OH)_2D_3$ on the $1,25(OH)_2D_3$ receptor's binding performances: $24R,25(OH)_2D_3$ has already been found to be only 1/2000 as effective a ligand as $1,25(OH)_2D_3$ (9) for the chick intestinal receptor system. Here, we would like to report for the first time a direct inhibitory effect of $24R,25(OH)_2D_3$ on the $1,25(OH)_2D_3$ chick intestinal receptor's binding properties for $1,25(OH)_2D_3$. We have studied the saturation analysis of $1,25(OH)_2D_3$ to its intestinal receptor in the absence or in the presence of a ten-fold excess of $24R,25(OH)_2D_3$; this ratio is reflective of the physiological vitamin D metabolites status in the birds (10) where the plasma concentration of $24,25(OH)_2D_3$ is 10X that of $1,25(OH)_2D_3$. We have found both a decrease in the intensity of positive cooperativity, as well as a 40% reduction of affinity of the receptor, without modification of its capacity. This result suggests that $24R,25(OH)_2D_3$ is a potentially important modulator of the $1,25(OH)_2D_3$ receptor's binding affinity.

MATERIALS AND METHODS

Vitamin D compounds

[^{3}H]$1,25(OH)_2D_3$ (sp. act. 85 Ci/mmole) was obtained from Amersham/Searle. $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ were a kind gift from Dr. Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ).

Animals

White Leghorn cockerels were obtained on the day of hatching (Pace-Setter, Anaheim, CA) and raised on a standard rachitogenic diet (11) (0.6% calcium 0.4% phosphorus) for two weeks, ad libitum: then they were raised on a normal diet containing normal levels of calcium and phosphorus (1.2% Ca, 0.7% phosphorus) and 2000 IU of vitamin D_3 per kg of diet for two additional weeks.

Crude chromatin preparation

The chicks were killed by decapitation; the duodenal loop was excised, and its contents flushed out with saline solution. It was then slit longitudinally and washed with a saline solution (0.9% NaCl). All the subsequent steps were performed at 0-4°C. The mucosa was scraped from the serosa with the aid of two chilled glass slides on an inverted petri dish over ice. Then the mucosa was homogenized in TED buffer (40% weight/volume) containing 300 μM PMSF and centrifuged at 5000 g for 10 minutes. The pellet containing the cell nuclei was resuspended in the same volume of TED-Triton (0.5%) buffer, vortexed and centrifuged at 5000 g for 10 minutes. Two additional washes with TED-Triton were followed by two other centrifugations. The final pellet was resuspended in the same initial volume of TED-PMSF.

Binding assays

Aliquots (100 μl) of the crude chromatin receptor preparations were incubated for 18-20 hours at 0-4°C with increasing concentrations of [^{3}H]-1,25(OH)$_2$D$_3$ ranging from 0.4 nM to 12 nM, in the presence or absence of a 200-fold excess of 1,25(OH)$_2$D$_3$ to determine both the specific and non-specific ligand binding. Other aliquots of the same crude chromatin preparation were incubated in the same condition with the same range of [^{3}H]1,25(OH)$_2$D$_3$ concentrations, in the presence of a 10-fold excess of 24R,25(OH)$_2$D$_3$ for each 1,25(OH)$_2$D$_3$ concentration. The non-specific binding of 1,25(OH)$_2$D$_3$ in those conditions was determined by a parallel incubation in the presence of a 200-fold excess of both 1,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$. The hormone bound to the receptor was then separated from the free ligand by the hydroxylapatite batch assay, as described previously (12).

Data treatment

The resulting data from these studies (see Figures 1 and 2) were analyzed according to the procedures of Sasson and Notides (14) which allows assessment of cooperative binding of a ligand in the absence or presence of a non-radioactive ligand which is also a weak competitor. Thus the conventional equilibrium dissociation constant, K_D, for the ligand [^{3}H]-1,25(OH)$_2$D$_3$ binding to its chick intestinal receptor (closed circles) was determined via Scatchard analysis (15) and the conventional Hill coefficient, n_H, which is a measure of the cooperativity of binding of this same ligand was determined from the slope of the Hill plot. The data generated in the presence of the weak competitor 24R,25(OH)$_2$D$_3$ (open circles) technically yields only an apparent K_D and apparent n_H for [^{3}H]-1,25(OH)$_2$D$_3$ since the concentration of the competing ligand was varied so that it was always no more than 10X the concentration of the [^{3}H]-1,25(OH)$_2$D$_3$ under study. As discussed by Sasson and Notides (14) this form of data analysis facilitates discrimination of a nonradioactive molecule such as 24R,25(OH)$_2$D$_3$ functioning both as a pure competitor to reduce the quantity of [^{3}H]-1,25(OH)$_2$D$_3$ bound and as an effector molecule to influence the receptor's cooperative [^{3}H]-1,25(OH)$_2$D$_3$-binding mechanism. Procsal *et al.* (9) have previously shown that 24R,25(OH)$_2$D$_3$ is only 1/2000 as effective a competing ligand for the 1,25(OH)$_2$D$_3$ receptor. The Hill equation (13) describing a cooperativity between two binding sites is: $[B] = [B_{max}][F]^{n_H}/K_D + [F]^{n_H}$ where B is the ligand specifically bound to the receptor, F is the free ligand, n_H is the Hill coefficient and K_D is a composite average of the dissociation constants of the two binding sites of the receptor. Values of n_H greater than one indicate a positive cooperativity, and lower than one a negative cooperativity. If n_H equals one, the Hill equation simplifies to become the same formulation as the Scatchard equation.

RESULTS AND DISCUSSION

The Scatchard plot of the saturation of a 40% PMSF crude chromatin preparation by increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ was concave downwards, indicating the expression of a positive cooperativity mechanism (fig. 1A, closed circles). By contrast, in presence of 24R,25(OH)$_2$D$_3$ (fig. 1A, open circles), the Scatchard plot was linear (r = -0.98). As a result,

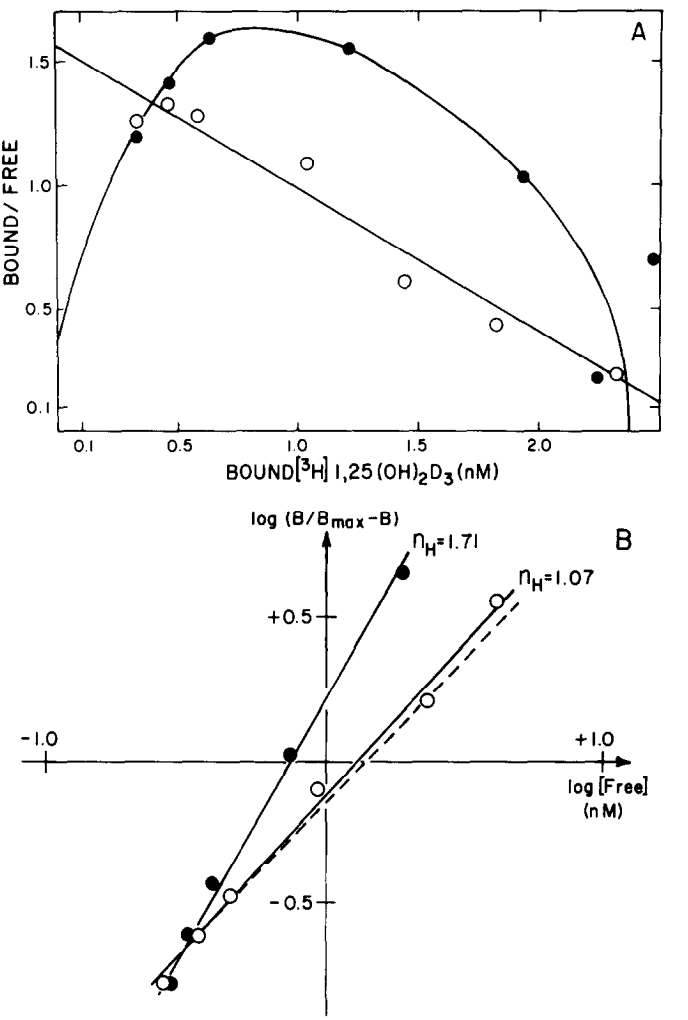

Fig. 1: Scatchard analysis (A) and Hill plot (B) of the saturation of a concentrated (40%) crude chromatin preparation of chick duodenal receptor by increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ in the presence or absence of a 200-fold excess of 1,25(OH)$_2$D$_3$. This crude chromatin was prepared from a group of 7 to 8 chicks raised on a normal diet supplemented with vitamin D$_3$. This analysis was made in the absence (●——●) or in the presence (○——○) of a 24R,25(OH)$_2$D$_3$ 10-fold excess for each concentration of [^{3}H]1,25(OH)$_2$D$_3$. The dashed line in fig. 1B represents a Hill plot with n_H = 1.00 (no cooperativity).

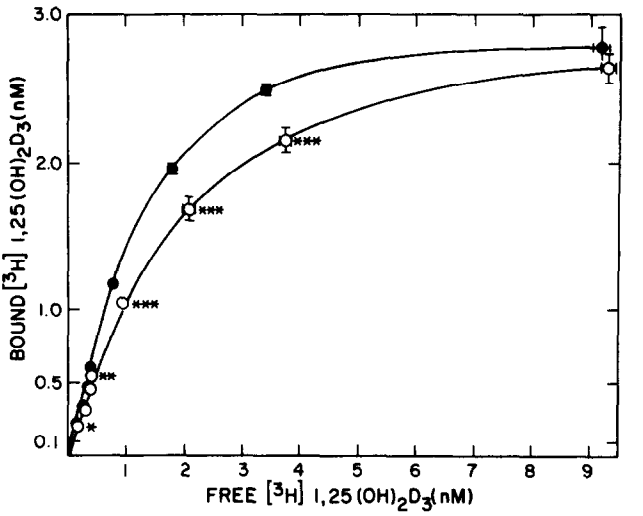

Fig. 2: Saturation analysis of 5 different crude chromatin preparations each resulting of a group of 7 to 8 chicks raised on a normal diet supplemented with vitamin D_3, by increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ in the absence (●——●) or presence (○——○) of a 24R,25(OH)$_2$D$_3$ 10-fold excess for each concentration of [^{3}H]1,25(OH)$_2$D$_3$. Each point represents the mean ±SEM of the specific binding. (*** $p < 0.01$; ** $p < 0.02$; * $p < 0.05$).

the Hill plot (fig. 1B) showed a very significant positive cooperativity (n_H = 1.71) in the binding of 1,25(OH)$_2$D$_3$ to this chromatin receptor preparation. In the presence of 24R,25(OH)$_2$D$_3$ (open circles), there was a drastic reduction in cooperativity (n_H = 1.07).

The saturation analysis (fig. 2) of 5 different batches of crude chromatin receptor, each of them prepared from a pool of 7 to 8 chicken duodena, showed a significant shift to the right of the 1,25(OH)$_2$D$_3$ binding curve in the presence of a 10-fold excess of 24R,25(OH)$_2$D$_3$ (open circles). At each sub-saturation concentration studied, there was a decrease in the binding of 1,25(OH)$_2$D$_3$ induced by the 10-fold excess of 24R,25(OH)$_2$D$_3$. There was no significant change in the maximum binding capacity (B_{max}) of the receptor as reported to the protein concentration (see table I), but the affinity was significantly ($p < 0.01$) reduced from 0.97±0.09 to 1.36±0.04, as well as the Hill coefficient ($p < 0.03$) from 1.49 to 1.26 in the presence of 24R,25(OH)$_2$D$_3$.

In conclusion, these results suggest that 24R,25(OH)$_2$D$_3$ may regulate the affinity of the 1,25(OH)$_2$D$_3$ intestinal receptor through a modulation of the positive cooperativity mechanism; this suggests that the intestinal

TABLE I

	B_{max}	K_D(nM)	n_H
Control	200±9	0.97±0.09	1.49±0.06
24R,25$(OH)_2D_3$	189±11 (N.S.)	1.36±0.04 ($p < 0.01$)	1.26±0.06 ($p < 0.03$)

Intestinal crude chromatin 1,25$(OH)_2D_3$ receptor's binding performances in the absence or presence of a 10-fold excess of 24R,25$(OH)_2D_3$ for each concentration of [^{3}H]1,25$(OH)_2D_3$ studied. The indicated values are the mean ±SEM of 5 different batches of crude chromatin, each of them constituted of 7 to 8 chicks. B_{max} is the maximum binding capacity in fmole/mg protein; K_D is the average apparent dissociation constant of the receptor's two binding sites extrapolated from the Hill plot (in nM) and n_H is the apparent Hill coefficient.

1,25$(OH)_2D_3$ receptor must have also a binding recognition site for 24R,25-$(OH)_2D_3$. This possibility is currently under investigation in our laboratory.

ACKNOWLEDGEMENT

This is paper LXI in a series entitled "Studies of the mode of action of calciferol"; reference (2) is the previous paper in this series. This study was supported, in part, by USPHS grant AM-09012-20. Francois Wilhelm was supported by a fellowship from Research and Industry, State Department, Paris, France.

REFERENCES

1. Tsai, H. C., and Norman, A. W. (1973) J. Biol. Chem. 248, 5967-5975.
2. Wilhelm, F., and Norman, A. W. (1984) Febs Letters 170, 239-242.
3. Wilhelm, F., and Norman, A. W. (1984) International Symposium on Osteoporosis, Copenhagen, June 3-8, 1984 (in press).
4. Henry, H. L. (1984) Vitamin D (Kumar, R. ed.) pp. 151-174, M. Nijhoff publishing.
5. Norman, A. W., Roth, J., and Orci, L. (1982) Endocrine Reviews 3, 331-366.
6. Corvol, M. T., Ulmann, A., and Garabedian, M. (1980) Febs Letters, 116, 273-276.
7. Henry, H. L., and Norman, A. W. (1978) Science 201, 835-837.
8. Somjen, D., Binderman, I., and Weisman, Y. (1983) Biochem. J. 214, 293-298.
9. Procsal, D. A., Okamura, W. H., and Norman, A. W. (1975) J. Biol. Chem. 250, 8382-8388.
10. Norman, A. W., Leathers, V. L., and Bishop, J. E. (1983) J. Nutr. 113, 2505-2513.
11. Norman, A. W., and Wong, R. G (1972) J. Nutr. 102, 1709-1718.
12. Wecksler, W. R., and Norman, A. W. (1979) Anal. Biochem. 92, 314-323.
13. Hill, A. V. (1910) J. Physiol. 40, 190
14. Sasson, S., and Notides, A. (1982) J. Biol. Chem. 257, 11540-11545.
15. Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660-672.