Cooperativity in the binding of 1α,25-dihydroxyvitamin D_3 to the chick intestinal receptor

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The binding of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] to its chick intestinal receptor does not fit well to the linear regression line of Scatchard's model ($r = -0.79$; dissociation constant, $K_d = 0.51$ nM). In fact, a concave 'hook' curve describes the data better. By using the Hill analysis the linear fitting was improved ($r = 0.99$), the K_d was found to be 0.14 nM and the Hill coefficient (n_H) 1.42, which indicates a positive cooperativity in the binding of 1,25$(OH)_2D_3$ to its receptor. Further we found that K_d and n_H are strongly correlated ($p < 0.001$). These data suggest the existence of two ligand binding sites located in subunits for the 1,25$(OH)_2D_3$ receptor.

Chick *Intestine* *1,25-Dihydroxyvitamin D_3* *Receptor* *Cooperative behavior* *Affinity*

1. INTRODUCTION

It has been generally assumed that the 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] intestinal receptor exhibits only one ligand binding site with a dissociation constant, calculated by the Scatchard method [1], to be in the range of $1-5 \times 10^{-10}$ M [2–4]. We would like to report new data which suggests for the first time that the chick intestinal receptor for 1,25$(OH)_2D_3$ exhibits two ligand binding sites with a possibility of positive cooperativity in the binding of 1,25$(OH)_2D_3$.

2. MATERIALS AND METHODS

2.1. *Vitamin D compounds*

1,25-[^{3}H]$(OH)_2D_3$ (spec. act. 85 Ci/mmol) was obtained from Amersham (Searle). Vitamin D_3 was obtained in pure crystalline form from Sigma. 1,25$(OH)_2D_3$ was a kind gift from Dr Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ).

2.2. *Animals*

White Leghorn cockerels were obtained on the day of hatching (Pace-Setter, Anaheim, CA) and raised on a standard rachitogenic diet [5] (0.6% calcium, 0.4% phosphorus) for two weeks, ad libitum; then they were raised for two additional weeks on a normal diet containing normal levels of calcium and phosphorus (1.2% Ca, 1.2% P). All the birds received during this period (2 weeks) 1.62 nmol/day of vitamin D_3 per os in ethanol:propanediol (1:1, v/v).

2.3. *Receptor 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 one of three different 1,25$(OH)_2D_3$ receptor preparations were made.

2.3.1. KTED extract

The mucosa was homogenized in TED buffer, 10%, w/v (10 mM Tris; 1.5 mM EDTA; 1 mM DTT; pH 7.4) containing 0.3 M KCl to extract

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both the cytosol and nuclear receptors for $1,25(OH)_2D_3$. After 10–12 strokes in a Potter-Elvehjem homogenizer, the preparation was centrifuged at 5000 × *g* for 10 min. The supernatant was then centrifuged at 100000 × *g* for 1 h. The clear cytosol supernatant was used for the ligand binding assays.

2.3.2. Crude chromatin

The mucosa was homogenized in TED buffer (20%, w/v) containing 0.25 M sucrose and centrifuged at 600 × *g* for 10 min. The pellet containing the cell nuclei was resuspended in 20% TED, homogenized and centrifuged at 1000 × *g* for 10 min. Two additional washes with the same volume of TED were followed by centrifugations at 1000 × *g* for 10 min. Finally, the nuclear receptor was extracted from the nuclear chromatin with the KTED buffer at a dilution of 20%.

2.3.3. Cytosol fraction

The TED supernatant fraction (from step b, above) containing cytosol, mitochondria and microsomes was centrifuged at 100000 × *g* for 1 h. The clear cytosol fraction was then used for the assays.

2.4. *Binding assays*

Aliquots (100 μl) of the three different receptor preparations were incubated for 18–20 h at 0–4°C with increasing concentrations of $1,25[^3H](OH)_2D_3$ ranging from 0.2 nM to 8 nM, in the presence or absence of a 200-fold excess of $1,25(OH)_2D_3$ to determine both the specific and non-specific ligand binding. The hormone bound to the receptor was then separated from the free ligand by the hydroxylapatite batch assay as in [6].

2.5. *Data treatment*

The Hill coefficient (n_H) was calculated from the slope of the Hill plot. The Hill equation [7] 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 K_d values 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 $n_H = 1$, the Hill equation simplifies to become the same formulation as the Scatchard equation.

3. RESULTS AND DISCUSSION

When we studied the saturation by $1,25[^3H](OH)_2D_3$ of the chick intestinal receptor extracted by KTED (fig.1A), we observed a poor correlation coefficient ($r = -0.79$) for the linear regression analysis of the studied ligand concentrations when calculated as in [1]. The non-specific binding accounted only for 12% of the total binding. However, we obtained a much better fitting of the data by utilizing a concave hook curve. This hook curve is characteristic of a positive cooperativity phenomenon between two binding sites of the receptor examined [8]. The asymptote of the second part of the curve extrapolates to $-1/K_{d2}$, with K_{d2} defined as the dissociation constant of the second binding site. K_{d2} was found to be 5×10^{-11} M, which is much lower than the K_d calculated by the Scatchard method (51×10^{-11} M). By using the Hill plot, we obtained a much better fitting (fig.1B) of the data ($r = 0.99$) to a linear relationship than by the Scatchard method (fig.1A). The average K_d of the two binding sites was calculated from the intercept of the linear regression curve with the ordinate axis and was found to be 14×10^{-11} M, which is 4 times lower than the K_d obtained by the Scatchard method.

Calculated from the slope of the linear line, n_H was found to be 1.41 which indicates an important positive cooperativity in the binding of $1,25(OH)_2D_3$ to the two binding sites of the receptor. To our knowledge this is the first report which indicates cooperativity of $1,25(OH)_2D_3$ binding to the intestinal receptor. When we represented these same data (fig.2) by plotting log (free ligand) on the abscissa *vs* $1,25[^3H](OH)_2D_3$ specifically bound to the receptor on the ordinate, we found an inflection point located approximately at 75% of the maximum binding value of this receptor (0.67 nM) which is consistent with a positive cooperativity. By contrast, another receptor preparation for $1,25[^3H](OH)_2D_3$, exhibiting no cooperativity ($n_H = 1.0$), displayed a saturation curve with an inflection point at half maximum binding, which is consistent with the interaction of $1,25(OH)_2D_3$ with only one binding site [9].

Previous binding data from this laboratory [10] were plotted as a straight line in a Scatchard plot. A better fit, however, is obtained using a concave

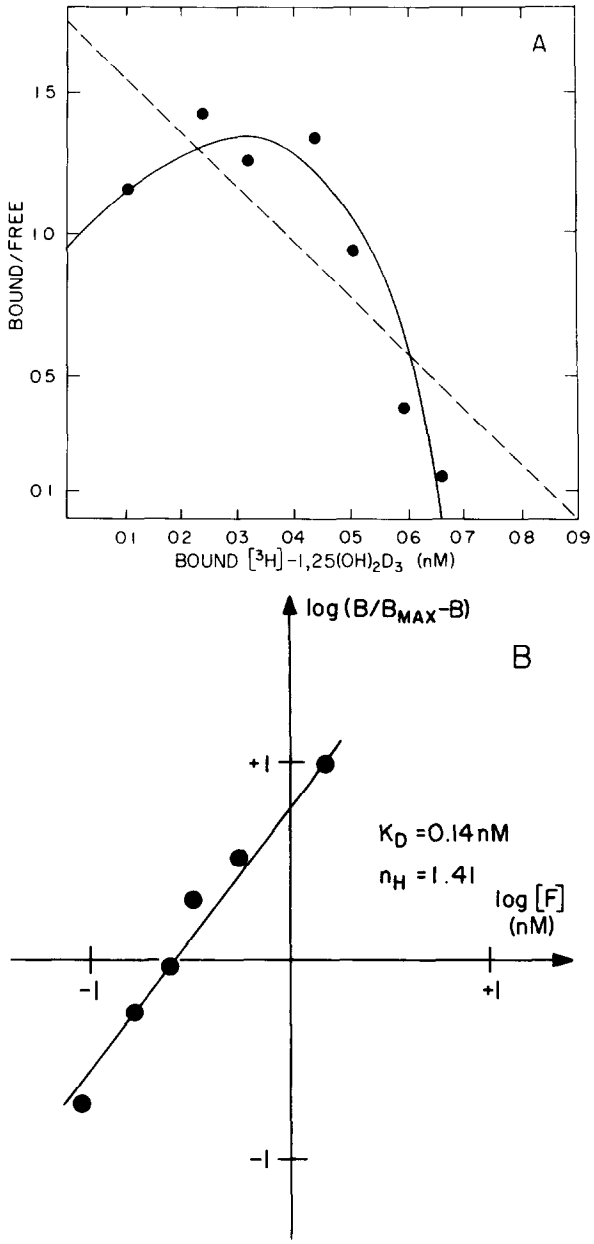

Fig.1. Scatchard analysis and Hill plot of the saturation of a chick intestinal receptor to $1,25(OH)_2D_3$ by increasing concentrations of $1,25[^3H](OH)_2D_3$ in the presence or in the absence of a 200-fold excess of $1,25(OH)_2D_3$. This receptor was extracted from an intestinal chromatin fraction by a high ionic strength buffer (0.3 M KCl in TED) from chicks raised on a normal diet (1.2% calcium, 1.2% phosphorus), supplemented with vitamin D_3 (1.62 nmol/day) for 2 weeks. The dashed line represents the calculated linear regression analysis utilizing all data points. The curved line represents the best fitting.

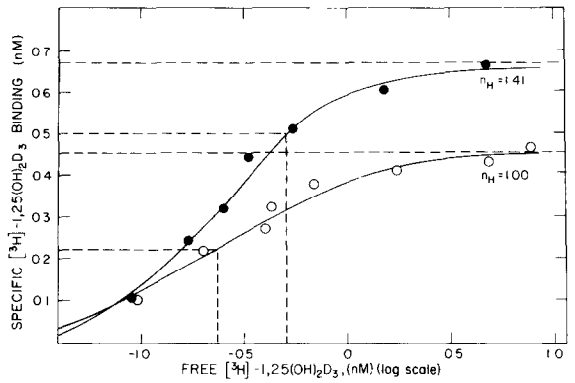

Fig.2. Saturation curves of two different chick intestinal receptors to $1,25(OH)_2D_3$, one with a highly positive cooperativity coefficient, n_H = 1.41 (●), the other exhibiting no cooperativity, n_H = 1.00 (○). These two receptor preparations were extracted by a high ionic strength buffer (0.3 M KCl in TED) from a chick intestinal chromatin preparation.

downward hook curve. Replotting these data using a Hill plot, we found a significant improvement in the correlation coefficient (r = 0.99) and an n_H of 1.34 ± 0.02 in crude chromatin preparations, indicating significant ($p < 0.01$) positive cooperativity in the binding of $1,25(OH)_2D_3$ to its receptor.

The present data show clearly that the $1,25(OH)_2D_3$ chick intestinal receptor can exhibit a

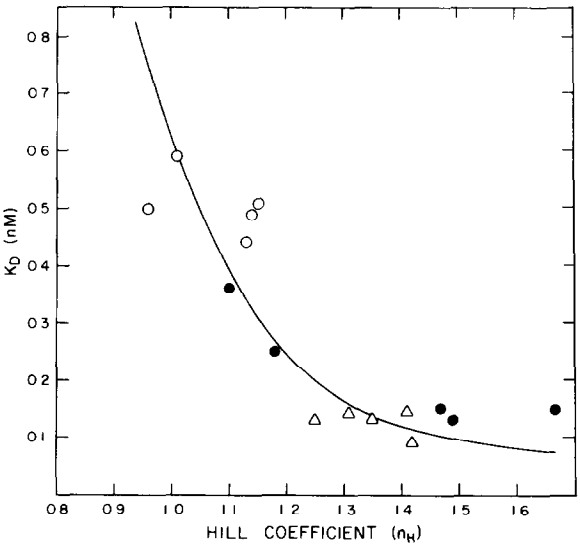

Fig.3. Relationship between K_d and n_H calculated for 14 different receptor preparations: cytosol (●), crude chromatin (○) and KTED extract (△).

positive cooperativity mechanism in the binding of $1,25(OH)_2D_3$ to two binding sites of the receptor. This is consistent with reports [11,12] describing two different molecular masses for the unoccupied and occupied $1,25(OH)_2D_3$ intestinal receptor. Recently a similar mechanism has been reported in the binding of estradiol to its uterine receptor [13].

To study whether this cooperativity in the binding of $1,25(OH)_2D_3$ to its receptor has an effect on modulating the receptor's ligand binding affinity, we plotted (fig.3) the values obtained for K_d and n_H in 14 different receptor preparations resulting from the three receptor isolation procedures (KTED preparation, crude chromatin, and cytosol) which were obtained from birds raised on a normal vitamin D-replete diet. We found a correlation coefficient of -0.83, which is highly significant ($p < 0.001$) between K_d and n_H. The best fitting was found to be a hyperbolic decreasing function; when the cooperativity increases, the observed K_d values variation decreases. Further we did not find a significant difference by the Fischer test in comparing the variances of K_d and n_H. These facts strongly suggest that the modulation of this cooperativity mechanism may play a key role in the variation of the $1,25(OH)_2D_3$ intestinal receptor affinity.

The more complete characterization of this cooperativity mechanism, its presence in other tissues and other species and its importance in the adaptation to diets deficient in calcium or in phosphorus is now in progress in our laboratory.

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