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Research Article

The dynamic equilibrium between in vivo occupied and unoccupied 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] receptors of the chick intestinal mucosa was investigated by the exchange assay previously reported [(1980). *J. Biol. Chem.***255:** 9534-9537]. These parameters and their correlation to biological response, i.e., the levels of intestinal vitamin D-dependent calcium binding protein (CaBP), were assessed under different physiological conditions. After a single 1,25$(OH)_2D_3$ injection (3.25 nmol), occupied receptor levels increased sharply to a maximum between 1 and 2 h, followed by a rapid decline. A single dose of 1α-hydroxy-vitamin D_3 [1α$(OH)D_3$], an analog that requires 25-hydroxylation for biological activity, resulted in a protracted, albeit lower, response with maximal receptor occupancy at 6 h and half maximal levels 24 h after injection. The intestinal receptor occupancy patterns mirrored the serum 1,25$(OH)_2D_3$ levels after either 1,25$(OH)_2D_3$ or 1α$(OH)D_3$ treatment. Additionally, time-course (half-life) of blood disappearance of 1,25$(OH)_2D_3$ and occupied receptor levels were similar (1.9 and 2.3 h, respectively), suggesting that the amount of occupied 1,25$(OH)_2D_3$ receptor is determined by a simple equilibrium between serum 1,25$(OH)_2D_3$ and unoccupied receptors. A dose-response study after intramuscular 1,25$(OH)_2D_3$ injection yielded a hyperbolic curve with an apparent plateau at 70% receptor occupancy, corresponding to 5 nmol 1,25$(OH)_2D_3$ injected. Half-maximal occupancy was reached after a dose of 1 nmol 1,25$(OH)_2D_3$, corresponding to 1.5 ng 1,25$(OH)_2D_3$/ml serum. From this value the apparent K_d […]

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ABSTRACT The dynamic equilibrium between in vivo occupied and unoccupied 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] receptors of the chick intestinal mucosa was investigated by the exchange assay previously reported [(1980). *J. Biol. Chem.* **255:** 9534–9537]. These parameters and their correlation to biological response, i.e., the levels of intestinal vitamin D-dependent calcium binding protein (CaBP), were assessed under different physiological conditions. After a single 1,25$(OH)_2D_3$ injection (3.25 nmol), occupied receptor levels increased sharply to a maximum between 1 and 2 h, followed by a rapid decline. A single dose of 1α-hydroxy-vitamin D_3 [1$\alpha$$(OH)D_3$], an analog that requires 25-hydroxylation for biological activity, resulted in a protracted, albeit lower, response with maximal receptor occupancy at 6 h and half maximal levels 24 h after injection. The intestinal receptor occupancy patterns mirrored the serum 1,25$(OH)_2D_3$ levels after either 1,25$(OH)_2D_3$ or 1$\alpha$$(OH)D_3$ treatment. Additionally, time-course (half-life) of blood disappearance of 1,25$(OH)_2D_3$ and occupied receptor levels were similar (1.9 and 2.3 h, respectively), suggesting that the amount of occupied 1,25$(OH)_2D_3$ receptor is determined by a simple equilibrium between serum 1,25$(OH)_2D_3$ and unoccupied receptors. A dose-response study after intramuscular 1,25$(OH)_2D_3$ injection yielded a hyperbolic curve with an apparent plateau at 70% receptor occupancy, corresponding to 5 nmol 1,25$(OH)_2D_3$ injected. Half-maximal occupancy was reached after a dose of 1 nmol 1,25$(OH)_2D_3$, corresponding to 1.5 ng 1,25$(OH)_2D_3$/ml serum. From this value the apparent K_d in vivo is 3.7 nM, which is similar to that determined in vitro. A 10-fold increase in the 1$\alpha$$(OH)D_3$ dose resulted in less than a doubling of the levels of serum 1,25$(OH)_2D_3$, occupied 1,25$(OH)_2D_3$ receptors, or CaBP. Under all experimental conditions, there was a positive correlation between occupied receptor and CaBP levels; however, the slope of the lines depended on the times chosen for the assays due in part to the lag period for CaBP induction and its accumulation within the cell. Conversely, the correlation between serum 1,25-$(OH)_2D_3$ levels and occupied receptor levels yielded a single regression line independent of the observation time. Short and long-term treatment with different vitamin D metabolites, estrogen, progesterone, or cortisol did not affect the levels of total intestinal 1,25$(OH)_2D_3$ receptor. Under normal physiological conditions, only 10–15% of the total 1,25$(OH)_2D_3$ receptor population was occupied by ligand. These studies provide a basis for further investigations of physiological and biochemical parameters of the vitamin D endocrine system and their clinical applications.

INTRODUCTION

1,25-Dihydroxyvitamin D_3 (1,25$[OH]_2D_3$),[1] an active metabolite of vitamin D_3, exerts at least part of its

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[1] *Abbreviations used in this paper*: CaBP, vitamin D-dependent calcium binding protein; DBP, vitamin D binding protein; DES, diethylstilbestrol; HAP, hydroxylapatite; 1$\alpha$$(OH)D_3$, 1$\alpha$-hydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; TED, 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol; TEDMo, TED + 10 mM Na-molybdate; TPCK, L-1-Tosylamide-2-phenyl-ethylchloromethyl ketone.

biological activity through an intracellular receptor system (1–3) similar to those of the other steroid hormones. Although 1,25-$(OH)_2D_3$ receptors have been described in a variety of animal and human tissues (4), the chick intestinal receptor has been the most extensively studied. In the past, characterization and quantitation of $1,25(OH)_2D_3$ receptors have been hampered by interference from the plasma-derived vitamin D binding protein, which is capable of binding $1,25(OH)_2D_3$ (5, 6). Recently we observed that unoccupied $1,25(OH)_2D_3$ receptors are predominantly localized in the chromatin fraction of hypotonic homogenates, whereas the vitamin D binding protein remains in the cytosol (7, 8). Therefore, assay of the chromatin fraction facilitates quantitation of unoccupied $1,25(OH)_2D_3$ receptors in the absence of the vitamin D binding protein (9).

Quantitation of unoccupied receptors is inadequate for assessing physiological function of the target tissues or the receptors, since only ligand-occupied receptors determine the physiological response. Therefore, we have recently developed an exchange assay for occupied $1,25(OH)_2D_3$ receptors that allows differential quantitation of both unoccupied and in vivo occupied receptors in chick intestinal mucosa (10). Similar assays in other steroid hormone receptor systems (11, 12) have provided invaluable information on the equilibrium between occupied and unoccupied sites in vivo, as well as the regulation of receptor levels by endogenous and exogenous factors (13, 14).

This paper provides the first evaluation of changes in the equilibrium between unoccupied and in vivo occupied $1,25(OH)_2D_3$ receptors under varying conditions of vitamin D status and other pertinent physiological perturbations. In addition, the physiological importance of occupied $1,25(OH)_2D_3$ receptors is established by comparison to the magnitude of the biological response.

METHODS

Animals and tissue preparation. Unless otherwise indicated, 1-d-old White Leghorn cockerels were obtained from Pace/Setter, Alta Loma, CA and were raised for 3–4 wk on a standard rachitogenic diet (15). Subsequent treatments in individual experiments are given in the figure legends. After decapitation, the small intestine was removed, stripped of contents, and washed at 4°C in 0.9% NaCl. All subsequent steps were performed at 4°C unless otherwise stated. The mucosa was collected by scraping with a glass slide and the scraping was thoroughly homogenized in 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol (TED) buffer (10% wt/vol, ref. 9) with 10–12 strokes in a glass/teflon homogenizer (motor driven pestle). After a low speed spin of the homogenate (5,000 *g*, 10 min), the chromatin pellet was washed three times with vigorous vortexing in the same volume of TED + 0.5% Triton X-100. Incomplete removal of cytoplasmic elements may interfere with the action of L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) in later steps of the procedure.

Quantitation of unoccupied and occupied receptors (10). The chromatin pellet (Triton X-100 washed) was resuspended (10% tissue, wt/vol) in TED buffer containing 10 mM Na molybdate (TEDMo) and 500 kallikrein inhibiting units (KIU)/ml Trasylol. Aliquots (200 μl) of this chromatin suspension were pipetted into polypropylene tubes containing 8 mM [^{3}H]$1,25(OH)_2D_3$ in the presence or absence of a 200-fold excess of $1,25(OH)_2D_3$ (final concentrations). These tubes were incubated at 4°C for 4 h before the addition of hydroxylapatite to determine the level of unoccupied receptors. Coincidentally, TPCK (30 mM in ethanol) was added to an appropriate amount of the remaining chromatin suspension to give a final concentration of 100 μM. After incubation at 4°C for 30 min, 200-μl aliquots were pipetted into tubes containing the steroid as above, followed by incubation at 37°C for 30 min to determine the level of in vivo occupied receptor. At the end of both incubations, 500 μl HAP suspension (50% vol/vol in TED) was added to the tubes and the incubation was continued for 15 min at 4°C with vortexing. Then 1 ml TED-Triton was added and the tubes were vortexed and centrifuged at 1,500 *g* for 5 min; the resulting pellets were washed three times with 1 ml TED-Triton. The radioactivity in the final pellets was extracted with 1 ml 100% ethanol at 30°C for 30 min; the supernatant was dried under a stream of air and counted in 8–10 ml toluene containing 5.0 g/liter tertiary butylphenyl-5-(4-biphenylyl)1,3,4-oxadiazole (Amersham Corp., Arlington Heights, IL).

Radioimmunoassay for vitamin D-dependent calcium binding protein (CaBP). Intestinal CaBP levels were determined by the method of Christakos and Norman (16) with the following modifications: all the buffers contained 0.5% bovine serum albumin, the incubation time with the antibody was reduced to 20 h, and 100 μl protein A (0.5% wt/vol) was substituted for the second antibody.

Serum $1,25(OH)_2D_3$ levels. This assay was performed by the method of Bishop et al. (17). Briefly, the serum was extracted with ether and subjected to high-pressure liquid chromatography. The region of $1,25(OH)_2D_3$ elution was pooled and quantitated by a competitive binding assay utilizing the chick intestinal $1,25(OH)_2D_3$ receptor.

Chemicals. $1,25(OH)_2[26,27\text{-}^3H]D_3$ (9 Ci/mmol) was prepared from Amersham Corp. 9 Ci/mmol $1,25(OH)[26,27\text{-}^3H]D_3$ by kidney homogenate hydroxylation (18). Unlabeled $1,25(OH)_2D_3$ and other vitamin D metabolites were the kind gift of Hoffmann-La Roche (Nutley, NJ). Hydroxylapatite was purchased from Bio-Rad Laboratories (Richmond, CA). Triton X-100, TPCK and the steroid hormones were obtained from Sigma Chemical Co., St. Louis, MO; Trasylol was purchased from Mobay Chemical Corp., New York; protein A was obtained from BRL, Bethesda, MD.

RESULTS

Occupied and unoccupied receptors at varying times after $1,25(OH)_2D_3$ injection. An injection of $1,25(OH)_2D_3$ (3.25 nmol s.c.) resulted in a rapid increase in the level of occupied $1,25(OH)_2D_3$ receptor to a maximum at 1–2 h (Fig. 1A). This increase in occupied receptor was accompanied by a concomitant, stoichiometric decrease in unoccupied $1,25(OH)_2D_3$ receptors (Fig. 1B, C). Subsequently, $1,25(OH)_2D_3$ re-

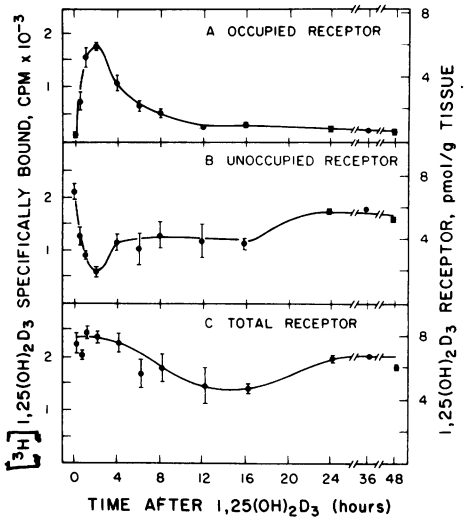

FIGURE 1 Occupied (A), unoccupied (B), and total (C) $1,25(OH)_2D_3$ receptors in the intestinal mucosa 0–48 h after s.c. injection of 3.25 nmol $1,25(OH)_2D_3$ in rachitic chicks. Data points are the mean±SEM of duplicate determinations in $n = 4$ individual chicks.

ceptor occupancy decreased rapidly to 38 and 15% maximal at 6 and 12 h postinjection, respectively; but the levels remained significantly elevated above preinjection levels through 48 h (Fig 1A). After 2 h, the unoccupied $1,25(OH)_2D_3$ receptor levels demonstrated a biphasic pattern: a rapid increase from the nadir at 2 h followed by a more gradual increase up to 24 h (Fig. 1B). During the time interval of 4–16 h after injection, the total number of $1,25(OH)_2D_3$ receptors (unoccupied plus occupied) decreased (Fig. 1C), suggesting that not all the previously occupied receptor was recycled to a form capable of rebinding $1,25(OH)_2D_3$.

Occupied and unoccupied $1,25(OH)_2D_3$ receptors at varying times after $1\alpha(OH)D_3$ injection. After $1\alpha(OH)D_3$ (6.5 nmol s.c.) injection, the occupied $1,25(OH)_2D_3$ receptor level (Fig. 2A) increased more slowly than after $1,25(OH)_2D_3$ injection. Maximal receptor occupancy was reached by 6 h, followed by a gradual decline. 24 h after $1\alpha(OH)D_3$ injection, the occupied receptor levels remained at half of the maximal level attained at 6 h, as compared with the lower (15% maximal) level sustained 24 h after $1,25(OH)_2D_3$ injection. The temporal pattern of serum $1,25(OH)_2D_3$ levels in these animals (Fig. 2B) was almost superimposable on that for the occupied $1,25(OH)_2D_3$ receptors, resulting in a highly significant correlation between these two parameters (Fig. 2C).

Dose of $1,25(OH)_2D_3$ injected vs. $1,25(OH)_2D_3$ receptor occupancy and CaBP[1] induction. Injection of increasing doses (0.1–10 nmol) of $1,25(OH)_2D_3$ resulted in increasing numbers of occupied $1,25(OH)_2D_3$ receptors at 2 h postinjection (Fig. 3A). A stoichiometric decrease in unoccupied receptor was observed (Fig. 3A); thus the total number of $1,25(OH)_2D_3$ receptors remained constant 2 h after all doses injected. There was an apparent plateau of 70% receptor occupancy at $1,25(OH)_2D_3$ doses > 5 nmol (Fig. 3B), resembling saturation curves for $[^3H]1,25(OH)_2D_3$ binding to the receptor in vitro. There was a highly significant ($P < 0.01$) correlation between the CaBP dose response 24 h after $1,25(OH)_2D_3$ injection and receptor occupancy 2 h postinjection (Fig. 3C).

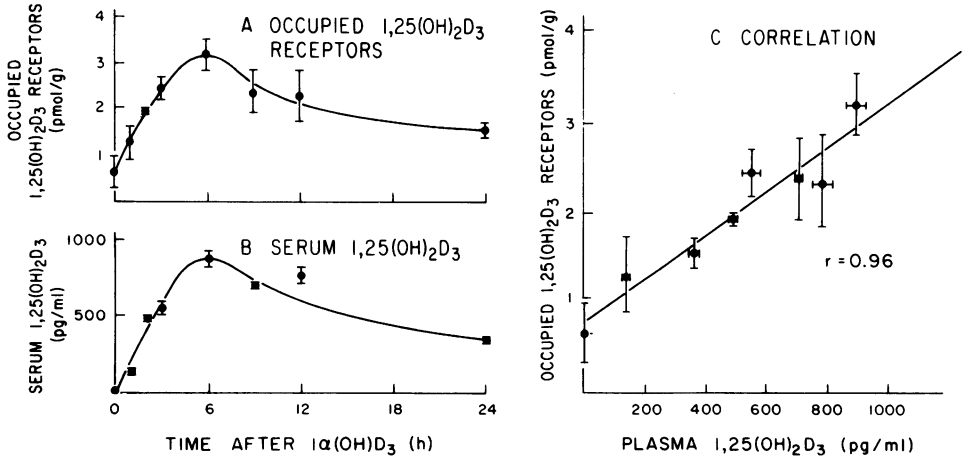

FIGURE 2 Time-course after $1\alpha(OH)D_3$. Occupied $1,25(OH)_2D_3$ receptors in intestinal mucosa (A), serum $1,25(OH)_2D_3$ levels (B), and the correlations between these parameters (C) at 0–24 h after s.c. injection of 6.5 nmol $1\alpha(OH)D_3$ in rachitic chicks. Data points are the mean±SEM of duplicate determinations in $n = 3$ individual chicks.

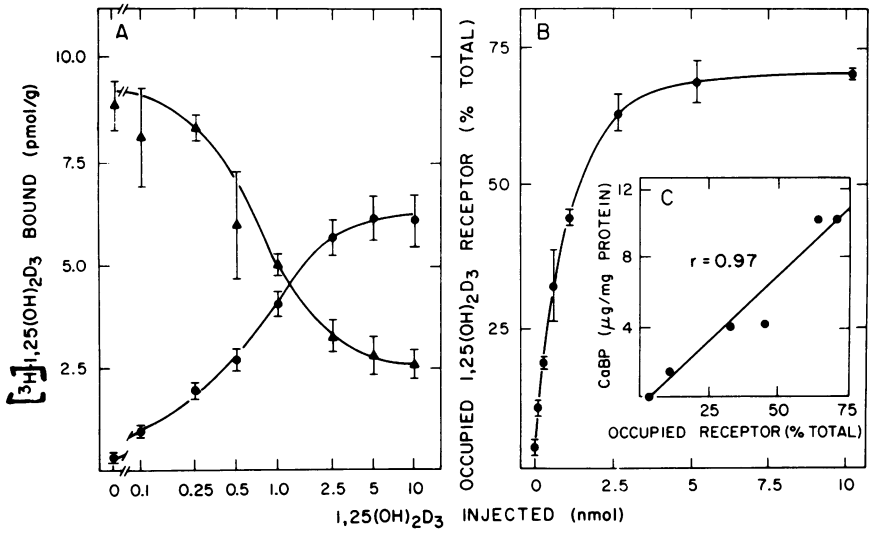

FIGURE 3 Unoccupied (A, ▲) and occupied (A, ●) $1,25(OH)_2D_3$ receptors and CaBP (B) in the intestinal mucosa of rachitic chicks after i.m. injection of 0–10 nmol $1,25(OH)_2D_3$. Receptor levels were determined 2 h after injection; CaBP was measured at 24 h. Data points represent the mean±SEM of duplicate determinations in n = 3 individual chicks. Also shown is the correlation between maximal (2 h) percent receptor occupancy and CaBP induction (24 h) for selected doses of $1,25(OH)_2D_3$ (C).

Dose of 1α(OH)D₃ injected vs. 1,25(OH)₂D₃ receptor occupancy. The levels of serum $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ receptor occupancy, and intestinal CaBP 24 h after injection of varying doses (2–200 nmol i.m.) of $1\alpha(OH)D_3$ are graphed in Fig. 4. Surprisingly, the slopes of all three curves were very shallow: a 10-fold increase in the dose of $1\alpha(OH)D_3$ resulted in only a doubling of the measured parameters. This muted response in all three parameters suggests that either the 25-hydroxylation or a slow release after injection are the rate-limiting steps for the biological action of $1\alpha(OH)D_3$. This fact, coupled with the reduced metabolic clearance rate of $1\alpha(OH)D_3$ as evidenced by the sustained plasma $1,25(OH)_2D_3$ levels (Fig. 2B), indicates that $1\alpha(OH)D_3$ can be considered a long-acting $1,25(OH)_2D_3$ analog.

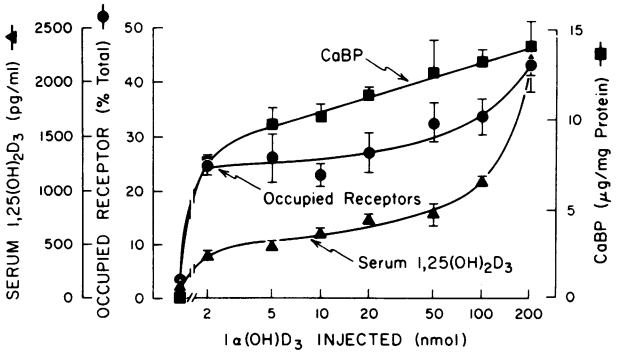

FIGURE 4 Occupied $1,25(OH)_2D_3$ receptors (●) and CaBP induction (■) in intestinal mucosa and serum $1,25(OH)_2D_3$ levels (▲) after i.m. injection of 0–200 nmol $1\alpha(OH)D_3$ in rachitic chicks. Occupied receptors and serum $1,25(OH)_2D_3$ levels were determined 6 h after injection; CaBP was assayed at 24 h. Data points represent the mean±SEM of duplicate determinations in n = 3 individual chicks.

Effect of vitamin D metabolites on 1,25(OH)₂D₃ receptor levels. Tissue responsiveness to estrogen or progesterone is regulated in part by a long-term increase (19) or decrease (14), respectively, in total receptor content after hormone administration. Thus we investigated whether vitamin D metabolites contribute to the regulation of intestinal $1,25(OH)_2D_3$ receptor levels. Since vitamin D deficient chicks were used, all changes in receptor levels can be attributed to the metabolite dosed and/or its in vivo derivatives. Daily treatment of chicks for 10 d with the indicated vitamin D metabolites resulted in different levels of occupied $1,25(OH)_2D_3$ receptors 24 h after the final injection (Fig. 5). In general, occupied receptor levels were higher in those animals treated with vitamin D derivatives that require one or more hydroxylations to generate a form with high affinity for $1,25(OH)_2D_3$ receptors. There was at least a 200-fold increase in the CaBP level with all the vitamin D forms; and there was a general correlation between the levels of CaBP and occupied receptor achieved with the metabolites. However, there was no effect of vitamin D treatment on the total tissue content of $1,25(OH)_2D_3$ receptors (Fig. 5). Similar data was obtained 24, 48, and 72 h

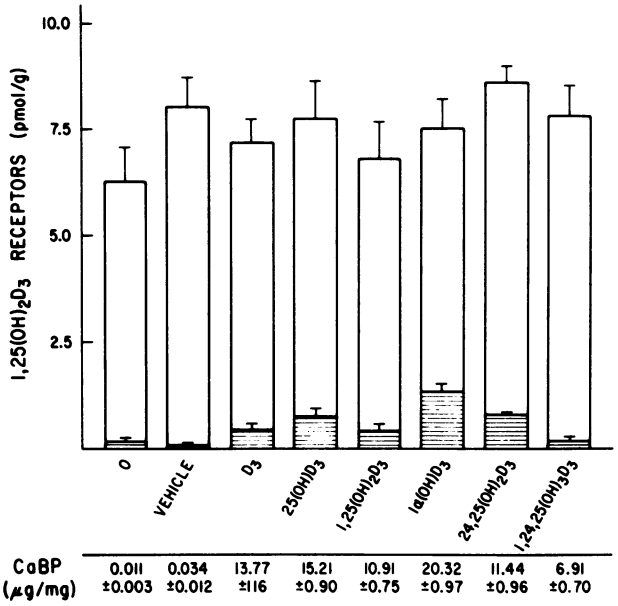

FIGURE 5 Effect of vitamin D metabolites on $1,25(OH)_2D_3$ receptor levels in the intestinal mucosa. Rachitic chicks were treated i.m. daily for 10 d with 3.25 nmol of the indicated metabolites and were killed 24 h after the last injection. Data points represent the mean±SEM of duplicate determinations in n = 8–10 individual chicks. The hatched bars represent occupied receptors, open bars represent unoccupied receptors, and the full bar height equals total receptor levels.

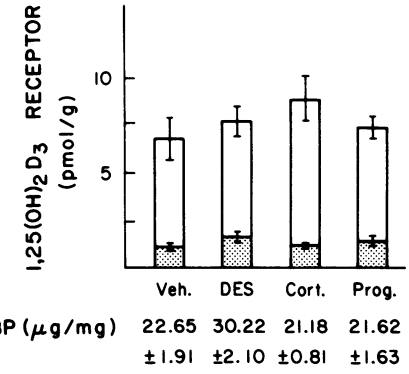

FIGURE 6 Effect of estrogen, progesterone, and cortisol on $1,25(OH)_2D_3$ receptor levels in normal chicks. Chicks were raised from hatch on a vitamin D-containing diet. After 3 wk they were injected i.m. daily for 8 d with vehicle (Neobee), 5 mg DES, 1 mg progesterone, or 0.5 mg cortisol and were killed 24 h after the last injection. Data points represent the mean±SEM of duplicate determinations in n = 6 individual chicks. Total bar heights represent total receptor levels, the hatched area represents occupied $1,25(OH)_2D_3$ receptors, and the open area represents the level of unoccupied receptors.

after daily treatment with these metabolites and 24, 48, and 72 h after a single injection of $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ (not shown). Thus these experiments suggest that the regulation of the total levels of the intestinal $1,25(OH)_2D_3$ receptor may not be directly related to the vitamin D status.

Effect of estrogen, progesterone, or cortisol on $1,25(OH)_2D_3$ receptor levels. Intestinal responses to vitamin D and/or $1,25(OH)_2D_3$ appear to be decreased by glucocorticoids (20–22) and to be increased by progesterone (23) and estrogens (23). We therefore investigated whether these effects could be mediated through effects on the levels of intestinal $1,25(OH)_2D_3$ receptors. The effect of daily treatment with diethylstilbestrol (DES) (5 mg), progesterone (1 mg), or cortisol (0.5 mg)[2] was evaluated in chicks raised on a vitamin D-containing diet (Fig. 6). Although the increase in occupied receptor levels after DES treatment was not statistically significant, it appeared to be biologically significant since CaBP levels were increased. This conclusion is consistent with its reported effects on circulating $1,25(OH)_2D_3$ (23–26) and previous reports of increased intestinal CaBP levels (23). However, there was no effect of any of these steroid hormones on the level of total $1,25(OH)_2D_3$ receptors in the intestinal mucosa (Fig. 6). Similar results were obtained after acute (24 h) and chronic (8 d) treatment in vitamin D-deficient chicks (not shown). These results are consistent with our inability to detect estrogen receptors in the intestinal mucosa of vitamin D-deficient chicks (data not shown), despite the enhanced vitamin D-dependent CaBP production in the presence of DES (Fig. 6).

Effect of reduced dietary Ca^{++} or phosphorus on $1,25(OH)_2D_3$ receptor levels. Under conditions of reduced dietary calcium or phosphorus, the activities of several aspects of the vitamin D endocrine system are stimulated (27–30). In order to determine whether these effects are mediated, in part, through regulation of intestinal $1,25(OH)_2D_3$ receptor levels, chicks were raised on vitamin D-containing diets with normal levels of Ca^{++} (1.1%) and P (0.75%), low calcium (0.2% Ca^{++}, 0.75% P), or low phosphorus (1.1% Ca^{++}, 0.25% P). Receptor occupancy was increased in the mucosa of chicks fed the low Ca^{++} diet (Fig. 7), in agreement with reports of increased intestinal $1,25(OH)_2D_3$ content (27, 28) and renal 25-hydroxyvitamin D-1α-hydroxylase stimulation (28) under these conditions. The total $1,25(OH)_2D_3$ receptor levels were lowered 25% ($P < 0.001$) by decreased dietary Ca^{++} and 46% ($P < 0.01$ vs. low Ca^{++} group) by reduced phosphorus (Fig. 7).

Correlations between levels of occupied $1,25-(OH)_2D_3$ receptors, intestinal CaBP, and serum

[2] The cortisol dose was diminished to reduce a high mortality rate encountered in previous experiments.

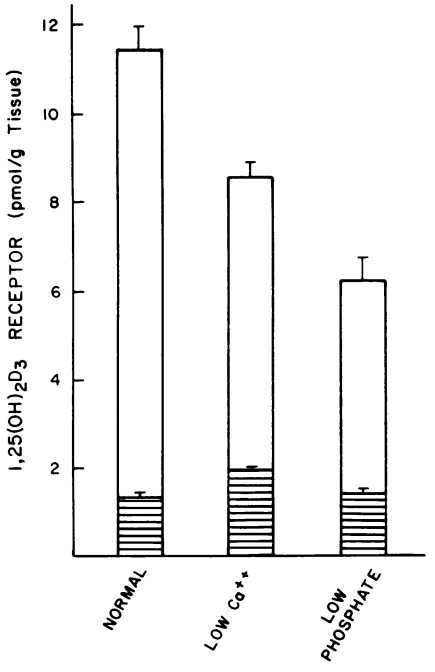

FIGURE 7 Effect of Ca^{++} or phosphorus deficiency on $1,25(OH)_2D_3$ receptor levels in the intestinal mucosa or normal chicks. Chicks were raised on vitamin D-containing diets with normal levels of Ca^{++} (1.1%) and phosphorus (0.75%), low calcium (0.2% Ca^{++}, 0.75% phosphorus), low phosphorus (1.1% Ca^{++}, 0.25% phosphorus). Data points represent the mean±SEM of n = 7–9 individual chicks. The hatched bars represent occupied receptors, open bars represent unoccupied receptors, and the full bar height equals total receptor levels.

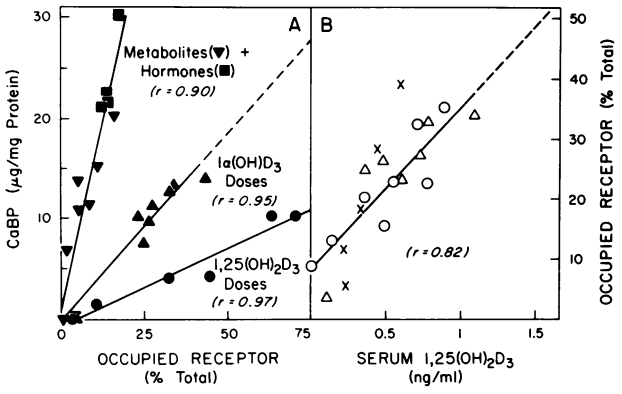

FIGURE 8 (A) Correlation between percent $1,25(OH)_2D_3$ receptor occupancy (2 h) and CaBP induction (24 h) after injection of varying doses of $1,25(OH)_2D_3$ (●) or $1\alpha(OH)D_3$ (▲) or after 10 d administration of varying vitamin D metabolites (▼) to rachitic chicks (data from Figs. 3, 4, 5, respectively) and after administration of other steroids to +D chicks (■). (B) Correlation between serum $1,25(OH)_2D_3$ and occupied $1,25(OH)_2D_3$ receptor levels at varying times after injection of 3.25 nmol $1,25(OH)_2D_3$ (×), 2 h after injection of 0–200 nmol $1\alpha(OH)D_3$ (○), or at varying times after 6.5 nmol $1\alpha(OH)D_3$ (△). Although the relationship between serum $1,25(OH)_2D_3$ levels and receptor occupancy is actually a hyperbola, approaching saturation at serum $1,25(OH)_2D_3$ levels greater than 2.5 ng/ml, the data at lower concentrations were treated as a simple correlation for the purpose of examining the relationships between the different treatments.

$1,25(OH)_2D_3$. For the purposes of comparison, the correlation between occupied $1,25(OH)_2D_3$ receptors and CaBP was determined from the data of Figs. 3–6. Each set of data resulted in a significant positive correlation, but different slopes were obtained (Fig. 8A). Further inspection of these data resulted in the conclusion that the level of occupied receptor at any time after $1,25(OH)_2D_3$ or $1\alpha(OH)D_3$ injection will correlate well with the CaBP response at 24 h. Thus at any time point after injection, the receptor occupancy will be determined by the dose administered; and any point on the receptor occupancy time-course reflects the entire shape of the curve at a given dose. Therefore, these data clearly imply a relationship between $1,25(OH)_2D_3$ receptor occupancy and the magnitude of CaBP induction. However, the data cannot answer the question of whether the CaBP response is determined by the level of receptor occupancy at a specific time or by the area under the curve, i.e., occupancy × time. Moreover, the slopes of the correlations will also be affected by the time selected for CaBP measurement, since $1\alpha(OH)D_3$ results in maximum receptor occupancy at a later time period and since the CaBP assay measures only accumulation within the tissue (i.e., synthesis minus degradation).

When serum $1,25(OH)_2D_3$ was compared to the level of occupied $1,25(OH)_2D_3$ receptors (Fig. 8B), there was a positive correlation which was independent of both dose (data from Fig. 4) and observation time (data from Figs. 1, 3). The latter observation was somewhat unexpected and leads to the calculation of the half-lives of serum $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ receptor occupancy after $1,25(OH)_2D_3$ injection, yielding almost identical half-lives (Fig. 9). These data suggest that the occupied $1,25(OH)_2D_3$ receptor exists in a simple thermodynamic equilibrium with serum $1,25(OH)_2D_3$ in vivo with no sustained receptor occupancy as serum $1,25(OH)_2D_3$ levels fall. This mechanism could result in acute regulation of Ca^{++} metabolism by providing a mode for rapid termination of biological response as serum $1,25(OH)_2D_3$ levels fall; however, full evaluation of this possibility awaits development of an assay for receptor retention at specific acceptor sites and/or sensitive CaBP mRNA assays.

DISCUSSION

The data presented herein provide the first analysis of functional, i.e., occupied $1,25(OH)_2D_3$ receptors

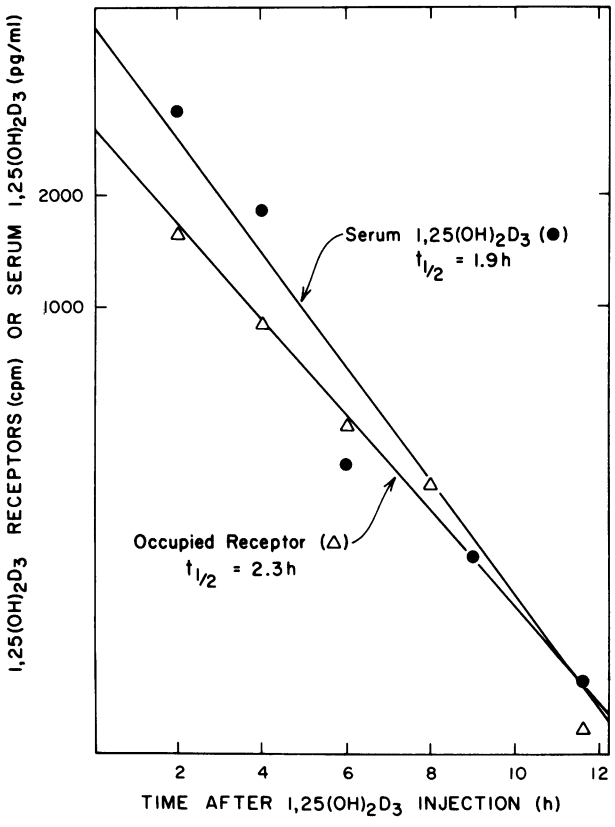

FIGURE 9 Half-life of $1,25(OH)_2D_3$ receptor occupancy (Δ) and of serum $1,25-(OH)_2D_3$ levels (●) after s.c. injection of 3.25 nmol $1,25(OH)_2D_3$ in rachitic chicks.

under varying physiological conditions. It is apparent that the recently reported method for quantitating in vivo occupied and unoccupied $1,25(OH)_2D_3$ receptors (10) can provide important information on vitamin D action at the receptor level. Such background studies are a necessary prerequisite to assessments of the vitamin D status of animals and humans and to the selection of clinical treatments for patients with disorders of Ca^{++} and phosphorus metabolism.

The temporal pattern of $1,25(OH)_2D_3$ receptor occupancy after $1,25(OH)_2D_3$ injection (Fig. 1) was similar to the profiles seen previously in other steroid hormone receptor systems (13, 31). The decrease in total $1,25(OH)_2D_3$ receptor levels seen 4–24 h postinjection is similar to the pattern obtained in the estrogen receptor system (19), but not the progesterone receptor system (14, 31). By analogy to the estrogen receptor system, this decrease could be interpreted as receptor inactivation coupled with a lag of *de novo* receptor synthesis; and it may represent a mechanism for terminating biological response to $1,25(OH)_2D_3$ by converting previously occupied receptors to a form incapable of rebinding ligand.

For all situations and at all times examined, the level of occupied $1,25(OH)_2D_3$ receptors was proportional to the level of serum $1,25(OH)_2D_3$ (Fig. 8B). Additionally, the half-lives of serum $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ receptor occupancy were strikingly similar (Fig. 9). These observations suggest that circulating $1,25(OH)_2D_3$ and the intestinal $1,25(OH)_2D_3$ receptors are coupled such that the physiological response is terminated immediately upon a drop in the level of circulating $1,25(OH)_2D_3$. This mechanism would not necessitate the same turnover of the receptors as implied for the mechanism proposed above. To our knowledge, the precise relationship between serum steroid and occupied receptor levels has not been reported in other systems. The simple coupling observed in the $1,25(OH)_2D_3$ system is very provocative; but its relationship, if any, to the protracted estrogen receptor retention time required to achieve the full estrogenic response (13, 32) is unclear. If the apparent differences are real, they may simply represent different requirements for minute-to-minute responses to the two hormones. The devastating physiological effects of relatively small changes in plasma Ca^{++} concentrations may demand a more immediate regulation of intestinal receptor occupancy and hence, intestinal Ca^{++} absorption.

The dose response of receptor occupancy after $1,25(OH)_2D_3$ injection (Fig. 3) displayed a hyperbolic saturation curve reaching an apparent plateau at 70% receptor occupancy (Fig. 3B). Whether the remaining 30% receptor population is unavailable for $1,25(OH)_2D_3$ binding is not clear. However, the general shape of this curve is similar to the saturation curves for $[^3H]1,25(OH)_2D_3$ binding to the receptor in vitro (7, 9). Extrapolating the available data comparing serum $1,25(OH)_2D_3$ levels and receptor occupancy (Fig. 8B), 50% in vivo receptor occupancy occurs at 1.5 ng $1,25(OH)_2D_3$/ml serum. Since the steroid concentration at half saturation reflects K_d, this data results in an estimated K_d = 3.7 nM for the receptor interaction in vivo. This value is in close agreement with the K_d (0.65–2.2 nM) determined in vitro (7). Thus, the serum vitamin D binding protein appears to have little influence on the availability of circulating $1,25(OH)_2D_3$ for receptor binding in vivo.

The finding that the total $1,25(OH)_2D_3$ receptor levels in the intestine were not affected/regulated even by drastic changes in levels of vitamin D analogs (Fig. 5) or by other steroid hormones (Fig. 6) was unexpected. These results suggest that the primary control of responsiveness in these physiological states occurs at the renal 25-hydroxyvitamin D_3-1-hydroxylase (28,

33) to regulate the circulating levels of $1,25(OH)_2D_3$. Therefore, it is important that in the normal state (vitamin D adequate) only 10–15% of the receptor is occupied (10), affording a maximum potential for responsiveness to elevated plasma $1,25(OH)_2D_3$ levels as dictated by the specific hormonal state of the animal. The existence of the simple coupled relationship between serum $1,25(OH)_2D_3$ and intestinal receptor occupancy (Results) provides a precise mechanism for response of this target tissue to acute changes in the renal 25-hydroxyvitamin D_3-1-hydroxylase activity. These observations provide substantiation for the physiological importance of mechanisms for acute regulation of the 1-hydroxylase activity (34).

The effects of severe dietary Ca^{++} or phosphorus restriction on total $1,25(OH)_2D_3$ receptor levels (Fig. 7) are interesting, but puzzling. In these deficiency states, one would predict that an increase in the $1,25(OH)_2D_3$ receptor level could improve intestinal responses to circulating $1,25(OH)_2D_3$ and, hence, contribute to homeostatic mechanisms to correct the mineral deficiency. In fact, the opposite response was seen. The mechanism of this effect, as well as the possibility that it may reflect an artifact of the adverse effects of the mineral deprivations rather than a homeostatic compensatory mechanism, is presently under investigation.

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