1,25-Dihydroxyvitamin D₃ Receptors

DIFFERENTIAL QUANTITATION OF ENDOGENOUSLY OCCUPIED AND UNOCCUPIED SITES*

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The method described herein provides differential measurement of either in vivo occupied or unoccupied receptors for 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the chick intestinal mucosa. The unoccupied receptors are quantitated in the chromatin fraction by incubation with [³H]1,25(OH)₂D₃ at 4°C for 4 h without interference from filled sites. Fully occupied receptors can be measured at 37°C for 30 min with greater than 90% recovery. However, a mixture of occupied and unoccupied receptors cannot be directly quantitated at 37°C because of the high degradation rate of the unoccupied receptors at elevated temperatures (up to 50% degradation) which results in a falsely low total receptor estimate. Importantly, preincubation at 4°C with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 100 μM) blocks the unoccupied 1,25(OH)₂D₃ receptor sites allowing subsequent quantitation of exclusively occupied receptors by “exchange” incubation with [³H]1,25-(OH)₂D₃ at 37°C for 30 min. With this exchange of [³H]1,25(OH)₂D₃ in the presence of L-1-tosylamido-2-phenylethyl chloromethyl ketone, there was no significant difference between total receptor levels in intestinal mucosa of chicks with fully occupied receptors (13 nmol of 1,25(OH)₂D₃ injected for 2 h) and the receptor levels in chicks with only unoccupied receptors (8.01 ± 0.46 versus 7.82 ± 0.56 pmol/g of tissue, respectively). Furthermore, the exchange assay gave an estimate of the level of occupied receptor identical with that obtained by direct extraction and quantitation of [³H]1,25(OH)₂D₃ present in the chromatin after in vivo [³H]1,25(OH)₂D₃ injection. Therefore, this exchange assay allows quantitative measurement of occupied 1,25(OH)₂D₃ receptor and/or unoccupied receptors in the chick intestinal mucosa. Additionally, L-1-tosylamido-2-phenylethyl chloromethyl ketone may be useful to block unoccupied sites in other steroid hormone systems. This 1,25(OH)₂D₃ receptor exchange assay will be important for physiological experiments in the vitamin D endocrine system and may be adaptable to tissues from other species of laboratory animals or from humans.

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), an active metabolite of the secosteroid vitamin D₃, is now generally considered to be a steroid hormone by classical criteria (1-3). Our recent data indicate that unoccupied 1,25(OH)₂D₃ receptors are present in crude chromatin fractions under low salt conditions in vitro (4). Consequently, quantitation of unoccupied receptor levels in the chick intestinal mucosa is best achieved by assay of the crude chromatin fraction (5). Due to the slow dissociation rate of 1,25(OH)₂D₃ from its receptor (4), only endogenously unoccupied 1,25(OH)₂D₃ receptors are detected by this method (incubation with [³H]1,25(OH)₂D₃ at 4°C for 4 h).

The assay of endogenously occupied sites in addition to the unoccupied receptors has proven very useful in other steroid hormone systems (6-9). These assays require exchange of the endogenous ligand against ³H-ligand in crude nuclear preparations in vitro. Since unoccupied 1,25(OH)₂D₃ receptors of the chick intestinal mucosa are also associated with the nuclear/chromatin fraction (4), differentiation between and quantitative measurement of the unoccupied and occupied receptors is more complicated. In this paper, we report a method for differential detection and quantitative measurement of either in vitro occupied or unoccupied 1,25(OH)₂D₃ receptors.

MATERIALS AND METHODS

Animals and Tissue Preparation—White Leghorn cockerels obtained on the day of hatch from Pace-Setter, Alta Loma, CA were raised for 3 to 4 weeks on a standard rachitogenic diet (10). 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 scraped with a glass slide and the scraping was thoroughly homogenized in TED (10% w/v Ref. 5) with 10 to 12 strokes in a glass/Teflon homogenizer (motor-driven pestle). After a low speed spin of the homogenate (5000 x g, 10 min), the chromatin pellet was washed three times with vigorous blending on a Vortex mixer in the same volume of TED + 0.5% Triton X-100. Incomplete removal of cytoplasmic elements interfered with the action of TPCK in later steps of the procedure (see below).

Quantitation of Unfilled and Filled Receptors—Since both the filled and unfilled receptors were in the crude chromatin fraction under the above conditions (4), only this subcellular fraction was needed for receptor quantitation. The chromatin pellet (Triton X-100-washed) was resuspended (10% tissue w/vol) in TED buffer containing 10 mM Na-molybdate and 500 KIU/ml of Trasylol. Molybdate stabilized the receptors at 4°C and 37°C, whereas Trasylol improved receptor stability only at 37°C (data not shown). Aliquots (200 μl) of the chromatin suspension were pipetted into polypropylene tubes containing 8 nM [³H]1,25(OH)₂D₃ (9 Ci/nmol, Amersham/Searle) in the presence or absence of a 200-fold excess of 1,25(OH)₂D₃ (final concentrations). These tubes were incubated at 4°C for 4 h to give 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 with

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containing the steroid as above, followed by incubation at 37°C for 30 min to determine the level of filled receptor. At the end of both incubations, 500 µl of hydroxyapatite suspension (50% v/v in TED) was added to the tubes, and the incubation was continued for 15 min at 4°C with blending on a Vortex mixer. Then, 1 ml of TED-Triton was added, and the tubes were blended on a Vortex mixer and centrifuged at 5000 x g for 5 min; the resulting pellet was washed three times with 1 ml of TED-Triton. The radioactivity in the final pellet was extracted with 1 ml of 100% ethanol at 30°C for 30 min; the supernatant was dried under a stream of air and counted in 8 to 10 ml of toluene containing 5.25 g/liter of tertiary butylphenyl-5-(4-biphenyl)-1,3,4-oxadiazole (Amersham/Searle). Hydroxylapatite was kindly given by Hoban-La Roche (Nutley, NJ). Hydroxylapatite was purchased from Bio-Rad (Richmond, CA). Triton X-100 and TPCK were obtained from Sigma Chemical Co., St. Louis, MO. Trasylol was purchased from Mobay Chemical Corp., New York, NY.

RESULTS

Binding Kinetics of Unfilled and Filled 1,25(OH)2D3 Receptors—Physical separation of filled and unfilled 1,25-(OH)2D3 receptors is impossible in chick intestinal mucosa due to the presence of unoccupied receptors in the crude chromatin fraction under low salt conditions in vitro (4). Therefore, we investigated whether filled and unfilled 1,25-(OH)2D3 receptors could be distinguished and quantitated through differential binding kinetics. The time and temperature dependency of [3H]1,25(OH)2D3 binding to unfilled receptors is shown in Fig. 1A. At 4°C, receptor binding increased for 4 h and reached a plateau thereafter with no noticeable degradation up to 24 h. At 23°C, maximum binding occurred at 30 min. Elevating the temperature to 30 or 37°C resulted in significant receptor degradation, allowing only detection of 80% and 70%, respectively, of the unfilled receptors present.

The binding kinetics of the in vivo filled receptors is presented in Fig. 1B. At 4°C, a rapid, albeit small, increase in [3H]-1,25(OH)2D3 binding (probably due to residual unfilled sites) was followed by a slow second binding component representing exchange of endogenous ligand against [3H]1,25(OH)2D3. Incubation at 37°C for 30 min resulted in the maximum level of exchange. Extrapolating the degradation rate at 37°C to zero time indicates that about 90% of the filled receptors were measured at 30 min. Taken collectively, these data show that the unfilled 1,25(OH)2D3 receptors can be readily measured at 4°C for 4 h with no influence from filled sites. Conversely, in a sample with both occupied and unoccupied receptors, the receptor level measured at 37°C for 30 min represents a value that is higher than that of the occupied sites. This value is also lower than the amount of total (filled and unfilled) 1,25(OH)2D3 receptors due to partial degradation of unfilled receptors at 37°C.

Occurring receptor binding sites with ligand in vitro can stabilize receptors for 1,25(OH)2D3 (11) as well as for other steroid hormone systems (12-14). Unfortunately, occupying endogenously unfilled 1,25(OH)2D3 receptor sites by preincubation with [3H]1,25(OH)2D3 at 4°C in vitro did not increase the receptor recovery during the subsequent elevation of temperature (data not shown).

TPCK Blocks [3H]1,25(OH)2D3 Binding to Unfilled Receptors—In studies to stabilize the receptors at elevated temperatures, we observed that TPCK (and TLCK in higher concentrations) inhibited binding of [3H]1,25(OH)2D3 to unoccupied receptors with no effect on occupied receptors at selected concentrations (Fig. 2). The interaction of TPCK with unoccupied receptors was quite rapid at 4°C, reaching completion within 10 min (Fig. 2A). Importantly, incubation at 4°C with TPCK did not affect the ligand bound to endogenously occupied receptors. At elevated temperatures, where steroid dissociates from the occupied receptors, only 500-fold higher TPCK concentrations inhibited the [3H]1,25(OH)2D3 exchange process (Fig. 2B). These data indicate that selection of the optimal concentration of TPCK is critical for the

![Fig. 1. Time and temperature dependency of [3H]1,25(OH)2D3 binding to unoccupied (A) and to in vivo occupied 1,25(OH)2D3 receptors (B).](image)

![Fig. 2. Time (A) and dose dependency (B) of TPCK effects on 1,25(OH)2D3 receptors.](image)
accurate measurement of filled sites and must be carefully established in each system. Under our conditions, preincubation with 100 μM TPCK for 20 to 30 min at 4°C effectively blocks all the unoccupied 1,25(OH)2D3 receptors with only a negligible effect (only 8% underestimation in Fig. 2B) on subsequent exchange of [3H]1,25(OH)2D3 for the endogenously occupied sites.

**Quantitation of Unfilled and Filled Receptors for 1,25-(OH)2D3**

Application of exchange conditions to physiological/endocrinological studies requires differential quantitation of unfilled and filled 1,25(OH)2D3 receptor sites in samples containing mixtures of these sites in different ratios. To validate the TPCK assay for physiological experiments, chromatin from the intestinal mucosa of vitamin D-deficient chicks (unfilled receptors) and 1,25(OH)2D3-replete chicks (occupied receptors) were mixed in known ratios and assayed as described under "Materials and Methods." This mixing experiment indicated that unfilled and filled receptor sites could be accurately measured at 4°C and at 37°C in the presence of 100 μM TPCK, respectively, whereas in the absence of TPCK the total receptor level is underestimated up to 2-fold (Fig. 3).

**Scatchard Analyses under Nonexchange and Exchange Conditions**—Under these incubation conditions, Scatchard plots of [3H]1,25(OH)2D3 binding to unoccupied and to occupied receptors were linear, indicating the presence of a single binding component for 1,25(OH)2D3 (Table I). Additionally, the $K_d$ values were in the expected range (0.2 to 1.5 nM). The higher $K_d$ value of the filled receptors at 37°C correlates with previously reported temperature effects on unoccupied receptors (15).

**Receptor Levels in Vitamin D3-deficient and in 1,25-(OH)2D3-replete Chicks**—If both unfilled and filled receptors are measured quantitatively, the same number of total receptors should be present in vitamin D-deficient chicks and in 1,25(OH)2D3-replete chicks. Thus, unfilled and filled 1,25(OH)2D3 receptor levels were determined in the intestinal mucosa of 1,25(OH)2D3-injected and noninjected vitamin D-deficient chicks. Importantly, there was no difference in the total receptor levels measured in the two groups of chicks (Table II). This result validates the receptor levels measured under nonexchange as well as under exchange conditions.

**Occupied Receptor Levels Estimated by Exchange or Ligand Extraction**—Filled receptor levels estimated by the exchange assay were compared with the level of extractable ligand after in vivo injection. For this experiment, vitamin D-deficient chicks were injected subcutaneously with 100 units of [3H]1,25(OH)2D3 (0.1 Ci/mmol) 2 h prior to killing. Chromatin was prepared as usual and was then subjected to the exchange assay or was extracted by the method of Bligh and Dyer (16). The lipid extract was chromatographed via standard high pressure liquid chromatography methods (17) and the area under the 1,25(OH)2D3 peak was counted for [3H]1,25(OH)2D3-replete chicks (mean ± S.E. for six observations).

**Table I**

| Receptor Status       | Vitamin D3-deficient | 1,25(OH)2D3-replete* |
|-----------------------|----------------------|----------------------|
|                       | $N$                  | $K_d$                |
|                       | $N$                  | $K_d$                |
| Unfilled (4°C)        | 5.5                  | 0.5                  |
| Filled (37°C + TPCK)  | ND$^*$               | ND$^*$               |

* Injected with 13 nmol of 1,25(OH)2D3 2 h prior to killing.

**Table II**

Comparison of receptor levels in chromatin of the intestinal mucosa of vitamin D3-deficient and of 1,25(OH)2D3-replete chicks (mean ± S.E. for 10 observations)

**Table III**

Level of filled and unfilled 1,25(OH)2D3 receptors in normal chicks (mean ± S.E. for six observations)

| Vitamin D3 status | Receptor content | Per cent filled |
|-------------------|------------------|-----------------|
|                   | Filled           | Unfilled        |                  |
|                   | pmol/g           | pmol/g          |                  |
| Deficient         | 0.16 ± 0.04      | 3.26 ± 0.48     | 4.6% ± 0.1       |
| Replete*         | 0.60 ± 0.08      | 4.72 ± 0.77     | 11.8% ± 0.6      |

* Chicks received 2000 units of vitamin D3/kg of diet/day to supplement the D-deficient diet fed to both groups.

† Significantly different (p < 0.001) from the -D group by Student's t test.
assay, we evaluated the levels of occupied 1,25(OH)₂D₃ receptors in chicks provided a maintenance dose of vitamin D₃. As shown in Table III, the level of occupied receptor in the normal chicks remained quite low, although they were significantly elevated above the levels in the vitamin D-deficient chicks. These results indicate that, as in other steroid hormone systems (7, 8), only a small fraction of the total 1,25(OH)₂D₃ receptor population is required for hormonal response. The difference in total receptor content in these two groups is currently under investigation.

**DISCUSSION**

The method described herein provides a means of quantitative detection of unoccupied and/or occupied 1,25(OH)₂D₃ receptors present in the chick intestinal mucosa. Unoccupied receptors can be readily measured at 4°C for 4 h with no noticeable influence from filled sites (Fig. 1). If all the receptors are occupied by ligand in vivo, they can be measured at 37°C for 30 min with about 90% recovery (Fig. 1B). Conversely, at 37°C only about 60 to 70% of the unoccupied receptors are detected due to the high rate of degradation of these sites at the elevated temperature. Occupying the unfilled 1,25(OH)₂D₃ receptors in vitro before increasing the temperature did not increase the number of these sites detected. These results may indicate a differential stability of in vivo and in vitro filled receptor. Importantly, measurement of a mixture of filled and unfilled sites at 37°C in the absence of TPCK gave a receptor estimate that is lower than the total receptor level present in the tissue but higher than the filled receptor level (Figs. 1A and 3).

In other steroid hormone systems, TPCK inhibits binding to the steroid binding site (18, 19), and protease substrates have been shown to compete for the binding of dexamethasone in HTC cells (20) and of estrone to α-gestoprotein.² Interestingly, preincubation with 100 μM TPCK at 4°C for 30 min completely blocked unfilled sites for 1,25(OH)₂D₃, without affecting endogenously occupied sites (Fig. 2). Surprisingly, during the subsequent temperature elevation, TPCK did not interfere with the exchange process (Fig. 2). Hence, differential quantitation of filled and unfilled 1,25(OH)₂D₃ receptors in chromatin of chick intestinal mucosa was achieved with this method (Fig. 3).

A problem associated with exchange assays is the possibility of receptor degradation at elevated temperatures which results in an underestimate of the filled receptor levels. Four independent lines of evidence show that the described exchange assay is quantitative. (a) Extrapolating the degradation rate at 37°C of in vivo filled receptor shows that 90% of the receptor is measured at 30 min (Fig. 1B). (b) Exchange of ligand at 4°C in presence of NaN₃ as previously described for the estrogen receptor (21) gave a 30% lower estimate of the total 1,25(OH)₂D₃ receptor than detected by the exchange assay developed herein.³ (c) The receptor level detected in a group of chicks dosed with 1,25(OH)₂D₃, where most of the receptor was occupied in vivo, is the same as the receptor value obtained in the vitamin D-deficient control group (Table III). (d) The amount of [³H]1,25(OH)₂D₃ extracted from chromatin after in vivo injection of [³H]1,25(OH)₂D₃ or of [³H]25(OH)₂D₃⁴ was equal to the amount of receptor measured by

³ M. E. Baker, C. S. Morris, and D. D. Fanestil, manuscript in preparation.

² W. Hunziker, M. R. Walters, and A. W. Norman, unpublished observations.

¹ A. Bar, M. R. Walters, W. Hunziker, and A. W. Norman, manuscript in preparation.

the exchange assay. Importantly, the exchange assay is much faster and less elaborate so that large numbers of samples can be easily processed.

This assay has shown that there is only a minimal difference in occupied 1,25(OH)₂D₃ receptors in animals of very different physiological states (Table III). This observation emphasizes the necessity for careful assessment of the exchange conditions in each system. Comparison of our results between different chick groups has resulted in the further conclusion that rigid controls will be necessary for each series of physiological studies in order to avoid erroneous conclusions.

The described method for differential measurement of unoccupied, occupied, and total receptor sites for 1,25(OH)₂D₃ in the chick intestinal mucosa should be an important tool for physiological studies in the vitamin D endocrine system. The procedure can be optimized for other target tissues and different species of laboratory animals. The small amounts of tissue required for the assay will be an asset in investigating disorders in the vitamin D system in humans. Additionally, TPCK may prove useful to distinguish between unoccupied and occupied receptor sites in other steroid hormone receptor systems.

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