Vol. 98, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1981 Pages 990-996

APPARENT NUCLEAR LOCALIZATION OF UNOCCUPIED RECEPTORS FOR 1,25-DIHYDROXYVITAMIN D_3*

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Received January 6, 1981

Summary

Previous studies have demonstrated that unoccupied 1,25-dihydroxyvitamin D_3 receptors are associated with crude chromatin under hypotonic conditions *in vitro*. The data presented herein show that unoccupied 1,25-dihydroxyvitamin D_3 receptors appear to be associated with chromatin prior to solubilization by dilution/homogenization in both high and low salt buffers. Additionally the unoccupied receptors are recovered nearly quantitatively from purified nuclei. These results suggest that unoccupied 1,25-dihydroxyitamin D_3 receptors may be localized within nuclei *in vivo*.

The seco-steroid 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] affects Ca^{++} metabolism through an endocrine mechanism similar to that of other steroid hormones (1). In general, unoccupied steroid hormone receptors are present in cytosol preparations even in hypotonic buffers (2,3). Conversely, under similar conditions unoccupied $1,25(OH)_2D_3$ receptors are predominantly found in crude chromatin fractions (4,5). Although these *in vitro* results cannot be readily extrapolated to the situation *in vivo* (4), the chromatin localization of unoccupied $1,25(OH)_2D_3$ receptors provides several important advantages including improved receptor quantitation in chick intestinal mucosa (5)

* This work was supported by USPHS grant AM-09012 and USPHS Training Grant AM-07310. This is paper XXIX in a series "Studies on the Mode of Action of Calciferol". All inquiries should be addressed to A. W. Norman.

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† Supported by a grant from the Swiss National Science Foundation.

Abbreviations used: $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; TD = 10 mM Tris, 1.0 mM dithiothreitol; TED = 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol; KTED = TED, 0.3 M KCl; 20% tissue weight/vol = 0.2 g tissue per ml homogenate.

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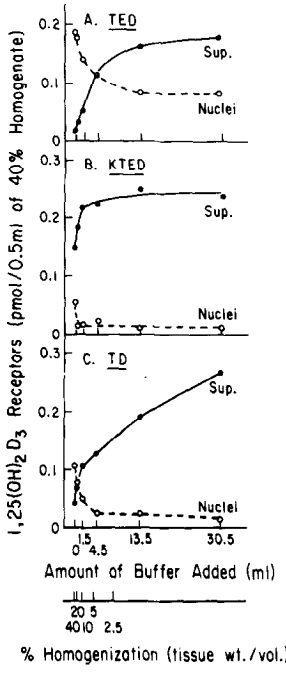


Figure 1. Effect of dilution on the apparent chromatin/cytosol distribution of unoccupied $1,25(OH)_2D_3$ receptors. Intestinal mucosa from vitamin D-deficient chicks was homogenized at 40% tissue weight/buffer vol in TED (A), KTED (B), or TD (C). Then buffer (volume as indicated) was added to separate 0.5 ml aliquots. After equilibration (4°C, 10 min) the samples were centrifuged (6000 rpm, 10 min). The supernatants were processed to cytosol (104,000 x g 1 h) after dilutions 1-3 were brought to 3 ml with buffer. The initial pellets were extracted with 3 ml 0.6 M KTED (4°C, 45 min) and centrifuged at 104,000 x g, 1 h. Receptor content of the cytosols and the chromatin extracts was assessed by incubation with 2 nM [^{3}H]$1,25(OH)_2D_3$ (82 Ci/mmol) $\pm$ excess $1,25(OH)_2D_3$ (4°C, 2 h), followed by the hydroxylapatite assay. For details of the experimental rational, see Sheridan et al. (8). In addition to the volume of buffer added, the per cent homogenization (tissue weight/vol) is given on the X-axis for comparison to dilutions in other systems.

and partial purification which facilitates receptor identification in tissues containing high amounts of the vitamin D binding globulin (4). The recognition of multiple parallels between the behavior of unoccupied $1,25(OH)_2D_3$ receptors and receptors for triiodothyronine, which are considered to be intrinsic nonhistone chromosomal proteins (6,7) has led us to pursue the question of the in vivo locus of the unoccupied $1,25(OH)_2D_3$ receptors.

Methods

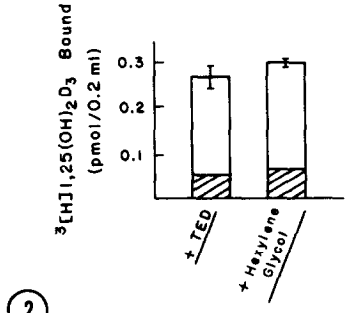
Animal treatments and the preparation of crude cytosol and crude chromatin have been described elsewhere (4,5). The details of the buffer dilution experiment (8) are given in the legend to Figure 1.

Nuclei were purified by the hexylene glycol procedure (9) as modified by Conn and O'Malley for use in the chick oviduct (10). Intestinal mucosa of vitamin D-deficient chicks was homogenized (10-12 strokes in a Dounce homogenizer) in the hexylene glycol buffer (0.5 M hexylene glycol, 1 mM PIPES, 10 M $CaCl_2$, 12 mM monothioglycerol, pH 7.4) at 20% tissue weight/vol. The homogenate was passed through four layers of cheesecloth and centrifuged at 900 x g for 5 min. To prepare cytosol, the supernatant was centrifuged at 104,000 x g for 1 h. The low speed pellet was washed three times by resuspending in the hexylene glycol buffer. Then the pellet was resuspended in 30 ml of sucrose buffer (2.0 M sucrose, 0.1 mM $MgCl_2$, 10 mM Tris, pH 7.5) and the nuclei were pelleted at 20,000 x g for 40 min. The purified nuclei were assayed by standard procedures (4,5) after resuspension in the hexylene glycol buffer or in TED$^{\$}$ (10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol). No difference in receptor concentration was observed in nuclei assayed in these two buffers when hydroxylapatite was added before the postincubation washes. The percent recovery of nuclei was calculated from the DNA content of the purified nuclei compared to the original homogenate. DNA was determined by the method of Burton (11).

RESULTS

Effect of Dilution on Receptor Distribution

Sheridan et al. (8) have postulated that unoccupied receptors for the classic steroid hormones are localized in nuclei in vivo and appear in cytosol preparations only because of the extensive dilutions usually inherent to homogenization techniques. We therefore assessed the effect of dilution on the chromatin/cytosol ratio of unoccupied $1,25(OH)_2D_3$ receptors both in a low salt (TED$^{\$}$) and in a high salt (KTED$^{\$}$) buffer. As shown in Fig. 1, dilution of the original homogenate markedly increased the solubilization of the unoccupied mucosal $1,25(OH)_2D_3$ receptors. The inclusion of 0.3 M KCl shifted the curve toward greater receptor solubilization at lower dilutions (Fig. 1B), an effect to be expected if ionic phenomena contribute to the forces which result in receptor localization in the nuclei (4). In TED, full solubilization of the unoccupied receptors into cytosol was not achieved (Fig. 1A). This result is consistent with our previous observations (unpublished) that EDTA reduces receptor losses from the chromatin during the washing process. The mechanism of the EDTA effect is unclear and is difficult to assess since omission of EDTA can result in a marked reduction in receptor recovery (Fig. 1C).



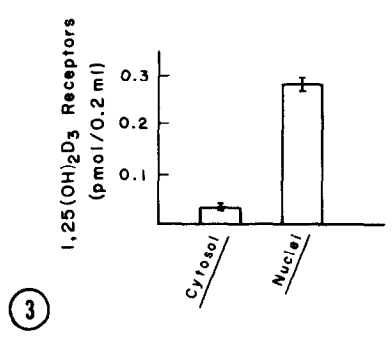


Figure 2. Stability of 1,25(OH)$_2$D$_3$ receptors in 0.5 M hexylene glycol. KTED cytosol was prepared from the intestinal mucosa of vitamin D-deficient chicks. Then 100 l of either TED or hexylene glycol (final concentration 0.5 M) was added prior to incubation with 5 nM [^{3}H]1,25(OH)$_2$D$_3$ in the presence (nonspecific binding, hatched bars) or absence of a 200-fold excess of 1,25(OH)$_2$D$_3$ (total binding, total bar height, mean ± SE of triplicate incubations.

Figure 3. Distribution of unoccupied 1,25(OH)$_2$D$_3$ receptors in hexylene glycol-derived cytosol and purified nuclei. Cytosol and purified nuclei were prepared from intestinal mucosa of vitamin D-deficient chicks by the hexylene glycol method. Aliquots of the cytosol and nuclei were incubated with 5 nM [^{3}H]1,25(OH)$_2$D$_3$ ± excess 1,25(OH)$_2$D$_3$ at 4°C for 18 h prior to the hydroxylapatite assay. The bars show the specific binding (mean ± SE of triplicate incubations).

Unoccupied 1,25(OH)$_2$D$_3$ Receptors in Purified Nuclei

In order to evaluate the possible *in vivo* locus of unoccupied receptors for 1,25(OH)$_2$D$_3$, we utilized the hexylene glycol method of nuclear purification (9,10). This procedure circumvents problems associated with perturbation of the system by ionic strength and by EDTA. Hexylene glycol itself does not affect receptor stability, as confirmed by its addition to KTED-cytosol prior to incubation with [^{3}H]1,25(OH)$_2$D$_3$ at 4°C for 18 h (Fig. 2).

Nuclei from vitamin D-deficient chick intestinal mucosa were prepared by the hexylene glycol procedure in 65 ± 12% yield as determined by DNA recovery. Under these conditions, the high nuclear/cytosol ratio previously observed for unoccupied 1,25(OH)$_2$D$_3$ receptors in crude preparations (4,5) was maintained (Fig. 3). This result suggested that these receptors may be localized within the nucleus *in vivo*. Scatchard analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding to the purified nuclei preparation yielded a K_d of 0.27 nM (Fig. 4),

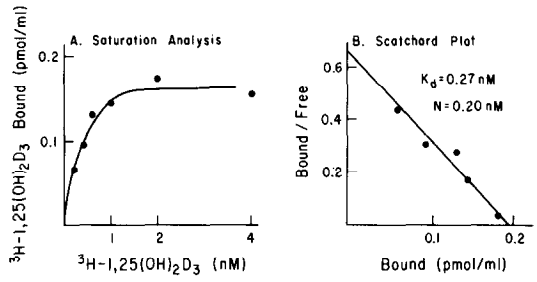


Figure 4. Saturation analysis (A) and Scatchard plot (B) of [^{3}H]1,25(OH)$_2$D$_3$ binding to purified nuclei. Purified nuclei were resuspended in TED and incubated with 0.2-10 nM [^{3}H]1,25(OH)$_2$D$_3$ ± excess 1,25(OH)$_2$D$_3$ at 4°C for 18 h prior to the hydroxylapatite assay.

confirming the identity of the binding sites in this subcellular fraction as unoccupied 1,25(OH)$_2$D$_3$ receptors. Comparison of the number of unoccupied 1,25(OH)$_2$D$_3$ receptors in purified nuclei to the total number in the tissue by TED-chromatin quantitation (5) indicated that at least 90% of the tissue complement of receptors were present in the purified nuclei when corrected for the differing DNA recoveries in these two procedures.

DISCUSSION

These data provide further evidence that unoccupied 1,25(OH)$_2$D$_3$ receptors have a high affinity for nuclear components (4). Additionally, the results suggest that unoccupied 1,25(OH)$_2$D$_3$ receptors may be localized in the nuclei *in vivo*. Of course, final confirmation of such nuclear localization must await methods for observing the receptor *in situ* in the absence of ligand.

The solubilization characteristics of unoccupied 1,25(OH)$_2$D$_3$ receptors are strikingly similar to those for triiodothyronine, which are presently considered to be integral nonhistone chromosomal proteins (6,7). These receptors can be partially extracted with STKM (0.25 M sucrose, 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$) buffer (12), are fully extracted in 0.4 M KCl (13, 14), and solubilize from nuclear preparations during incubation periods with labeled triiodothyronine (15). Importantly, Sheridan *et al.* (8) recently revived the hypothesis that unoccupied receptors for steroid hormones are also located within nuclei *in vivo*. Their conclusions were based on the

experimental design mimicked in Fig. 1 and on re-assessment of autoradiographic data (8). If this hypothesis is true, a lower affinity nuclear association of unoccupied estrogen, progesterone, and glucocorticoid receptors could lead to their solubilization *in vitro* even under mild conditions, resulting in the generalization of their cytosolic origin.

Taken collectively, these observations suggest that the nonmembrane-bound hormone receptors are all closely related, with $1,25(OH)_2D_3$ and ecdysteroid (16) receptors representing intermediate states between the more soluble steroid hormone (estrogen, progesterone, glucocorticoid) receptors and the most tightly chromatin-bound triiodothyronine receptor. Thus we favor the hypotheses that for all these receptors a large proportion of the total unoccupied receptor pool may be localized in the nucleus *in vivo*. The precise partitioning between nucleus and cytosol, in each instance, may be determined by the "local" ionic strength in that compartment of the intact cell. It is not possible, at this time, to predict the subcompartmentalization within the nucleus, i.e nucleoplasm, nuclear matrix, random chromatin association or specific chromatin association.

Sheridan *et al.* (8) recently proposed an equilibrium model for the nuclear/cytosol distribution of unoccupied steroid hormone receptors. This model suggests that the observed nuclear/cytosol ratio of steroid hormone receptors results only from the relative free water volumes of the two compartments. Additionally, these investigators proposed that precipitation of filled receptors onto chromatin simply results in a re-equilibration of the remaining unfilled receptors -- hence translocation. However, due to the differences in subcellular distribution *in vitro* of all these unoccupied receptors and recent data on the effects of ionic strength on $1,25(OH)_2D_3$ receptor distribution (Fig. 1 and ref. 2), we have modified this model to include a factor accounting for the ionic attraction of the unoccupied receptors for nuclear components (17). With such a revision, the receptor distribution model would predict the relative solubilization of either

unoccupied or occupied receptors or a mixture thereof, in any buffer utilized; and translocation would occur as a result of re-equilibration due to transformation of the receptors to a form with a higher affinity for DNA or chromatin. Studies are currently in progress to examine various aspects of this model.

ACKNOWLEDGMENTS

The authors wish to thank Ms. June Bishop for expert technical assistance in certain aspects of this study.

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