Unoccupied 1,25-Dihydroxyvitamin D₃ Receptors

NUCLEAR/CYTOSOL RATIO DEPENDS ON IONIC STRENGTH*

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Previous failures to detect cytosol receptors for 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in low ionic strength buffers were reassessed, since these buffers are used routinely with other steroid hormones. In the present studies, crude nuclei or chromatin fractions contained 90% of the tissue unoccupied 1,25(OH)₂D₃ receptors when the intestinal mucosa of vitamin D-deficient chicks was homogenized in low salt buffer (TED; 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol). Significant numbers of these receptors (25 to 50%) were also present in these subcellular fractions when the tissue was homogenized in higher ionic strength (100 to 300 mM) buffers. This property was not simply the result of generalized sticking of acidic proteins to nuclear components, since the acidic 25(OH)₂D₃-binding protein was not present in crude nuclear preparations. The unoccupied nature of the 1,25(OH)₂D₃ receptor sites in the intestinal mucosa of vitamin D-deficient chicks was confirmed by their extractability with high ionic strength buffers, by Scatchard analysis ($K_s = 0.65$ to 0.97 nM), and by gel filtration (Sephacryl S-200) chromatography. The proportion of unoccupied 1,25(OH)₂D₃ receptors associated with nuclear components varied inversely with the ionic strength of the buffers. Conversely, omission of sucrose from a buffer routinely used in such studies to stabilize nuclei had no effect on the cytosol/nuclear ratio. Unoccupied 1,25(OH)₂D₃ receptors were predominantly (61 to 92%) associated with nuclear components after TED homogenization in all tissues studied: chick intestinal mucosa, kidney, and pancreas; and an osteoblast-like mouse bone cell line. Although the subcellular localization of unoccupied 1,25(OH)₂D₃ receptors in vivo remains unresolved, the nuclear association in low salt buffer in vitro has many important biochemical and physiological ramifications.

1,25-Dihydroxyvitamin D₃, an active metabolite of the secosteroid vitamin D₃, is now generally considered to be a steroid hormone by all the classical criteria (1–5). Cytoplasmic receptors for this hormone have been described in multiple target tissues: chick, rat, and human intestinal mucosa (6–11), chick and human parathyroid gland (12, 13), chick kidney and pancreas (14), chick bone (15), and rat and mouse bone cell preparations (16, 17). In depth biochemical studies in the intestinal mucosa of rachitic chicks have described the subcellular localization of "$[{\text{H}}]1,25(\text{OH})_2\text{D}_3$" administered in vivo or in vitro (18–20), cytosol to nuclear translocation of the receptor upon ligand administration in vivo and in vitro (19, 21), and in vitro activation of the receptor (21, 22).

Interestingly, however, the presence of cytosol receptors for 1,25(OH)₂D₃ in tissues of rachitic animals has required "stabilization" of the receptors by intermediate (STKM) or high (KTED) ionic strength buffers (6–17). In contrast, other steroid hormone receptors are readily observed in cytosol prepared in very low ionic strength buffers (23, 24). In this report we demonstrate that in very low ionic strength buffer (TED) the unfilled 1,25(OH)₂D₃ receptors are located in the crude nuclear or crude chromatin subcellular fraction, with very little unfilled receptor remaining in the cytosol. Importantly, even cytosol preparations in KTED and STKM overlook residual authentic receptors for 1,25(OH)₂D₃ which are associated with the nuclear debris and which may have biochemical and physiological relevance (25).

MATERIALS AND METHODS

Animals and Tissue Preparation: Chick Intestinal Mucosa—White Leghorn cockerels obtained on the hatch date from Pace/Setter, Alto Loma, CA, were raised for 3 to 4 weeks on a standard rachitogenic diet (26). After decapitation, the duodenal loop was rapidly removed, stripped of contents, and washed at 4°C in 0.9% NaCl. All subsequent steps were performed at 4°C. The mucosa was scraped from the serosa with a glass slide and the Scraping was homogenized in the desired buffer (5 to 10% w/v) with 10 strokes in a glass-Teflon homogenizer. After a low speed spin (5000 x g, 10 min) of the homogenate, cytosol was centrifuged by centrifuging at 105,000 x g for 1 h. Nuclei or chromatin was prepared by washing the initial pellet three times in TED or in TED with 0.5% Triton X-100, respectively, and both preparations were resuspended in TED for incubation. Chromatin preparations necessitated higher force spins: 10,000 to 20,000 x g, 10 to 15 min. When desired, chromatin extracts were prepared by exposing the crude chromatin to STKM or KTED buffer (same volume as homogenization buffer) at 4°C for 45 min with frequent blending on a Vortex mixer. The residual pellet was recovered after centrifugation (5000 x g, 10 min) and the supernatant (extract) was cleaned of debris (105,000 x g, 1 h).

Assay for Unoccupied 1,25(OH)₂D₃ Receptor—The receptor content of each subcellular fraction was assessed by incubating 200 µL

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1 The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; TED buffer, 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol, pH 7.4 at 22°C; TED buffer, 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol, pH 7.4 at 22°C; TED buffer, TED, + 0.3 mM KCl, STKM buffer, TKM + 0.25 mM sucrose; TED:MO buffer, TED + 10 mM Na₂MoO₄; 10 mM TKM buffer, 10 mM Tris, 25 mM KCl, 5 mM MgCl₂, pH 7.4 at 22°C; D, 1.0 mM dithiothreitol added to any of the above buffers: 25(OH)D₃, 25-hydroxyvitamin D₃, T₃, triiodothyronine, 3',5'-triiodothyronine.
aliquots in polystyrene test tubes (12 x 75 mm) with 5 nM 1,25(OH)2D3 (26,27-3H)D3 (9 Ci/mm01) in the presence or absence of 200-fold excess unlabeled 1,25(OH)2D3 at 4°C for 90 min or 18 to 24 h. For assay of cytosol and soluble chromatin extracts, the incubation was terminated by the addition of 500 µl of 50% v/v hydroxyapatite (BioRad:Bio-Gel HTP) in TED and further incubation at 4°C for 15 min with frequent blending on a Vortex mixer. Although not strictly necessary, the addition of hydroxyapatite to chromatin pellets decreases the possibility of accidental pellet losses during the postincubation washes (25). In all cases, bound and free hormone were separated by washing the pellets three times with TED plus 0.5% Triton at 5000 x g. Then radioactivity was extracted with 1.0 ml of 100% ethanol at 30°C for 30 min with blending on a Vortex mixer for scintillation counting. 5 ml of Amersham’s ACS, Specific [3H]1,25(OH)2D3 binding was calculated by subtracting nonspecific binding ([3H]1,25(OH)2D3 bound in the presence of excess 1,25(OH)2D3 from total [3H]1,25(OH)2D3 binding.

Receptor Assay in Chick Kidney and Parathyroid Gland—The procedures outlined above for animal preparation and assay of unoccupied 1,25(OH)2D3 receptors were essentially unchanged for experiments with subcellular fractions of the chick kidney. However, in order to allow hypertrophy of the parathyroid glands, some chicks were kept for 5 to 6 weeks prior to killing. No other modifications were necessary for assay of receptors in the parathyroid.

Receptor Assay in Tissues of Vitamin D-deficient Rats—Weanling male rats (Sprague-Dawley) were obtained from Hilltop Lab Animals, Inc., Chatsworth, CA, and were placed on a synthetic nonrachitogenic diet (0.47% calcium, 1.2% phosphate) vitamin D-deficient diet (5,27) for 7 to 9 weeks prior to excision. Small intestinal mucosa, kidney, and testes were removed and washed in 0.9% NaCl. All tissues were resuspended in TED + 10 mM Na2MoO4 (33% v/v) prior to homogenization. Cytosol and crude chromatin were prepared as described above; chromatins was resuspended in TED for incubation. Aliquots (200 µl) were incubated at 4°C for 2 h with 2 nM 1,25(OH)2D3 (26,27-3H)D3 (82 Ci/mm01) ± 300-fold excess 1,25(OH)2D3; all tubes contained 50 nM nonradioactive 25(OH)D3. Specific receptor binding was assessed by the hydroxyapatite assay as described above.

Chemicals—1,25(OH)2D3 (26,27-3H)D3 was prepared from Amersham-Searle’s 9 Ci/mm01 25(OH)2D3 (26,27-3H)D3, by kidney homogenate hydroxylation (26). 1,25(OH)2D3 (23,24,25,26,27-3H)D3 (82 Ci/mm01) was obtained directly from Amersham-Searle, Arlington Heights, IL. Unlabeled 1,25(OH)2D3 and 25(OH)D3 were the kind gifts of Hoffmann-LaRoche (Nutley, NJ). Hydroxyapatite was purchased from Bio-Rad, Richmond, CA. Triton X-100 was obtained from Sigma Chemical Co., St. Louis, MO. The S-200 gel for gel filtration chromatography was purchased from Pharmacia, Piscataway, NJ.

RESULTS

Cytosol and Nuclear Distribution of Mucosal 1,25(OH)2D3 Receptors in TED and KTED—As a basis for subsequent comparisons, Table I summarizes the amounts of 1,25(OH)2D3 receptors detected in various cytosol preparations from the intestinal mucosa of vitamin D-deficient chicks. Very little, 1,25(OH)2D3 receptor is found in cytosols prepared in TED or TED plus 20% glycerol; and STKM cytosol contains less receptor than KTED cytosol. Due to the relatively gentle incubation conditions (4°C, 90 min) and the presence of a saturating level of ligand, receptor instability or degradation might not account for the differences between TED and the more frequently utilized buffers TED and STKM. Additionally, the effect of STKM seems unrelated to possible stabilization of receptor proteins by the sucrose, since the addition of glycerol to TED gave no substantial improvement in cytosol receptor detection.

To examine the tissue distribution of unoccupied 1,25(OH)2D3 receptors more fully, intestinal mucosa of vitamin D-deficient chicks was lightly mixed in TED and then half the tissue pool was homogenized in either TED or KTED. Cytosol and crude nuclei were prepared from each TED or KTED sample. Aliquots (200 µl) were incubated with 1,25(OH)2D3 as described under “Materials and Methods.” Surprisingly, a large number of unoccupied 1,25(OH)2D3 receptors were found in the nuclear preparations in both buffers (Fig. 1A). Additional studies have shown that similar numbers of receptors are present in crude chromatin prepared by washing the homogenization pellet three times with TED + 0.5% Triton X-100. Therefore, further experiments were performed on crude chromatin preparations to diminish the possibility of contamination with cytoplasmic components.

The above observations could have resulted simply from a generalized sticking of acidic cytoplasmic components to nuclear contents (28, 29). To test this possibility, the relative contamination of nuclear components by the vitamin D-binding globulin was assessed. This acidic protein (30), which binds 25(OH)D3 with high affinity, is prominent in plasma and is also present in many cytosol preparations (31–34). When [3H]1,25(OH)2D3 binding was measured in mucosal cytosol and chromatin prepared in TED, only 12% of the specific 25(OH)D3 binding was observed in the chromatin fraction (Fig. 1B). These data suggested that the nuclear association of the unoccupied 1,25(OH)2D3 receptors results from a specific biochemical property of the receptor molecules.

Unoccupied Nature of the Nuclear 1,25(OH)2D3 Binding Sites in Vitamin D-deficient Chicks—Although the chicks used in these experiments were fed only a rachitogenic diet (26) for 3 to 4 weeks prior to killing, the presence of endogenous metabolites bound to the receptors could have explained the relatively high affinity for chromatin. In order to test this possibility, rachitic chicks were untreated or were injected subcutaneously with 500 units of 1,25(OH)2D3 2 h prior to killing. TED-chromatin was prepared and was incubated with 5 nM [3H]1,25(OH)2D3 ± 1 µM unlabeled 1,25(OH)2D3 at 4°C for 2 or 24 h. As shown in Fig. 2, very little [3H]1,25(OH)2D3 exchanged with the endogenous 1,25(OH)2D3 bound to receptors in injected chicks under these incubation conditions, confirming the unfulfilled nature of the 1,25(OH)2D3 receptors in the TED-chromatin of intestinal mucosa from uninjected rachitic chicks. Additionally, this evidence that the residual binding sites in chromatin can be filled in vivo indicates that the [3H]1,25(OH)2D3 binding is not simply a non-receptor artifact of the in vitro chromatin preparation.

Characteristics of Residual Nuclear Unoccupied 1,25(OH)2D3 Receptors—The chromatin-associated [3H]1,25(OH)2D3 binding sites could represent a non-receptor moiety which was nevertheless inhibitable by excess 1,25(OH)2D3. Thus, we tested whether these sites could be extracted from the chromatin and still retain their [3H]1,25(OH)2D3 binding capability. Cytosol and chromatin from the intestinal mucosa of vitamin D-deficient chicks were prepared in TED or STKM; then the chromatin preparations were re-extracted with TED and KTED, and “Materials and Methods.” [3H]1,25(OH)2D3 binding was assessed in these cytosol and chromatin extracts by incubation at 4°C for 3 h followed by
25(OH)D3 specifically binding (duplicates not shown for clarity). TED and aliquots were fully homogenized in TED or in KTED. Then intestinal mucosa cytosol and nuclei (TED washed) or chromatin (TED X100 washed) were prepared. Specific binding was determined in those preparations by incubation with 5 nM \([\text{\{}^3H\text{\}}1,25(OH)\text{D}_3\text{\}}\) at 4°C for 18 h. The full bar heights represent total \([\text{\{}^3H\text{\}}\text{ligand binding}}\right)\) of duplicate determinations; the cross-hatched areas represent nonspecific binding (duplicates not shown for clarity).

Fig. 2 (center). Lack of exchange at 4°C of \([\text{\{}^3H\text{\}}1,25(OH)\text{D}_3\text{\}}\) with receptors occupied by nonradioactive 1,25(OH)\text{D}_3 after in vivo hormone administration. Vitamin D-deficient chicks were injected with 500 units of 1,25(OH)\text{D}_3 or with the vehicle (0.2 ml ethanol/pyropylene glycol 1:1) 2 h prior to killing. Mucosal scrapings were homogenized in TED and crude chromatin was prepared. Then aliquots were incubated with 5 nM \([\text{\{}^3H\text{\}}1,25(OH)\text{D}_3\text{\}}\pm 1 \mu M 25(OH)\text{D}_3\) (B) at 4°C for 18 h. The full bar heights represent total \([\text{\{}^3H\text{\}}\text{ligand binding}}\right)\) of duplicate determinations; the cross-hatched areas represent nonspecific binding (duplicates not shown for clarity).

The identity of the 1,25(OH)\text{D}_3 binding sites associated with TED-chromatin was explored by gel filtration chromatography. STKM extracts of TED-chromatin or KTED extracts of TED-chromatin were incubated with 5 nM \([\text{\{}^3H\text{\}}\text{ligand binding}}\right)\) and in the KTED extract of the chromatin. Data are expressed as the mean ± S.E. of triplicate determinations.

Effect of Buffer Ionic Content on Subcellular Distributions of the Unoccupied 1,25(OH)\text{D}_3 Receptors. The difference in the distribution of unoccupied 1,25(OH)\text{D}_3 receptors in KTED as opposed to TED can be readily explained by the differences in their ion concentration. Indeed, 0.3 to 0.4 M KCl can extract many intrinsic nuclear components (36–38), including in vivo occupied steroid hormone receptors (39, 40) and triiodothyronine receptors (41, 42). However, the situation is not as clear for the buffer STKM, where the ionic concentration is only 80 mM in spite of the high osmolarity. We therefore questioned whether specific chemical components of this buffer solution might cause the observed receptor distribution through some property other than ionic strength. The most likely single components for such effects were the divalent cation Mg\textsuperscript{2+}, which exerts multiple effects on DNA and nuclei (37), and sucrose which may preserve nuclear integrity (43), thus also preserving the true in vivo receptor distribution. In separate experiments mucosal scarpings of vitamin D-deficient chicks were mixed in TED or in TKM prior to complete homogenization of aliquots in TED, TED ± 5 mM MgCl\textsubscript{2}, and KTED or in STKM and TKM, respectively. The addition of 5 mM MgCl\textsubscript{2} to TED did not result in substantial receptor solubilization into the cytosol. Additionally, the exclusion of sucrose from STKM had no effect on the cytosol:chromatin distribution of the unoccupied 1,25(OH)\text{D}_3 receptors (not shown).
creased as an inverse of the ionic concentration of the homogenization buffer. This relationship is presented more accurately in the inset to Fig. 7, where the per cent of unoccupied 1,25(OH)₂D₃ receptors remaining on the chromatin is shown to vary inversely with the logarithm of the calculated ionic strength (I) of each buffer over the range I = 0 to 100 mM. The close relationship between the numbers of extracted receptors and the ionic strength of the buffer coupled with the specific lack of effect by sucrose suggests that low ionic strength buffers may more accurately represent the location of unoccupied 1,25(OH)₂D₃ receptors in vivo.

Lack of 1,25(OH)₂D₃ Receptor Binding Activity in Highly Purified Brush Border Membranes—Other investigators have demonstrated that crude chromatin prepared by methods similar to those used herein may be contaminated with brush border membrane components (44, 45). In order to differentiate between hypothetical 1,25(OH)₂D₃ binding components from this subcellular organelle and classical hormone receptors more specifically associated with nuclei, [³H]-1,25(OH)₂D₃ binding was assessed in highly purified brush border membranes (46) prepared in 30% yield by a modification of the methods of Rasmussen (47) and of Mircheff and Wright (48). After resuspension in TED at dilutions equivalent to 20% homogenate and 160% homogenate, 200 µl aliquots were incubated at 4°C for 18 h in the presence of 5 nM [³H]1,25(OH)₂D₃ alone (O—O) or with excess 25(OH)D₃. Then 500 µl of TED-Triton were added and the samples were eluted with 1,25(OH)₂Ds receptors remaining on the chromatin is shown to vary inversely with the logarithm of the calculated ionic strength (I) of each buffer over the range I = 0 to 100 mM. The close relationship between the numbers of extracted receptors and the ionic strength of the buffer coupled with the specific lack of effect by sucrose suggests that low ionic strength buffers may more accurately represent the location of unoccupied 1,25(OH)₂D₃ receptors in vivo.

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hydroxylapatite assay was continued to completion. Under these assay conditions, the specific $[^{3}H]_{1,25}$(OH)$_2$D$_3$ binding detectable in brush border membranes was negligible, even when the membranes were incubated at much higher tissue concentrations than those used for the chromatin preparations herein. Note that this experiment does not necessarily disprove the presence of specific $[^{3}H]_{1,25}$(OH)$_2$D$_3$ binding components in brush border membranes; however, it does ensure that these sites, if present, would not be detected by the hydroxylapatite assay as defined herein.

Cytosol and Nuclear Distribution of Unoccupied 1,25-(OH)$_2$D$_3$ Receptors—

The question of whether the nuclear association of unoccupied 1,25(OH)$_2$D$_3$ receptors is unique to chick intestinal mucosa is important in establishing the physiological and biochemical relevance of this observation. Therefore, the cytosol and nuclear distribution of 1,25(OH)$_2$D$_3$ receptors in TED buffer was assessed in parathyroid gland and kidney of rachitic chicks, in intestinal mucosa, kidney, and testes of vitamin D-deficient rats, and in an established osteoblast-like cell line from mouse calvaria (49). As shown in Table II, in all these tissues the majority (81.7 ± 0.4% S.E.) of the unoccupied 1,25(OH)$_2$D$_3$ receptors were associated with the chromatin preparation upon homogenization in TED. Thus, the nuclear association of unoccupied 1,25(OH)$_2$D$_3$ receptors results from an intrinsic property of the receptors or of the vitamin D hormone system, rather than a property of chick intestinal mucosal preparations.

**DISCUSSION**

These data clearly demonstrate that unoccupied 1,25(OH)$_2$D$_3$ receptors are associated with nuclear and chromatin fractions of target tissues homogenized in low salt buffers similar to those buffers employed in studying other steroid hormone receptors (23, 24). Indeed, the relative proportion of the unoccupied 1,25(OH)$_2$D$_3$ receptors in the chromatin decreases with the calculated ionic strength of the homogenization buffer (Fig. 7). These results raise the question of whether previously reported cytosol 1,25(OH)$_2$D$_3$ receptors represent instead receptors closely associated with nuclear components which are solubilized at the ionic strengths (100 to 300 mM) of the buffers originally used for tissue preparation. That the presence of unoccupied 1,25(OH)$_2$D$_3$ receptors in nuclei is not unique to chick intestinal mucosa (Table II) emphasizes the fact that this phenomenon may represent an inherent property of the 1,25(OH)$_2$D$_3$ receptor, or its endocrine system, or both, and is not a peculiarity of the behavior of mucosal tissue per se.

There is ample evidence throughout the literature that discrete receptors for the seco-steroid hormone 1,25(OH)$_2$D$_3$ exist in multiple target tissues (1-5). Thus, these binding sites for 1,25(OH)$_2$D$_3$ are saturable (6-17) and tissue specific (6, 7, 21, 22) and demonstrate hormone specificities for vitamin D analogs which agree with known biological potencies (3, 21, 50-55). Additionally, 1,25(OH)$_2$D$_3$ response curves for biological activities closely parallel receptor occupancy in vivo (2, 19, 56). Importantly, the nuclear-associated 1,25(OH)$_2$D$_3$ receptors described herein conform to these same criteria. The binding sites are saturable in vivo (Fig. 2) and in vitro (Fig. 6; Ref. 25) and exhibit hormone specificity (Figs. 1B and 5). These nuclear-associated unoccupied receptors also exhibit target tissue specificity since they are not present in nuclei or chromatin prepared from the liver of vitamin D-deficient chicks.

The co-identity of the nuclear-associated unoccupied 1,25(OH)$_2$D$_3$ receptors with previously described cytosol receptors is suggested by their solubilization from chromatin by the buffers commonly used for cytosol preparation (Figs. 3 and 4), by the similarity of the gel filtration patterns of cytosol and of chromatin-extracted receptors (Fig. 4), and by the similarity of their Scatchard binding characteristics with respect to linearity, $K_a$, and the number of receptor binding sites (Fig. 6). Comparison of the total quantities of 1,25(OH)$_2$D$_3$ binding sites in cytosol plus nuclei (or chromatin) after tissue homogenization in TED, KTED, or STKM provides further support for this concept (25). Even chromatin prepared from tissues homogenized in KTED retains a significant number of...
(25 to 30%) of the tissue unoccupied 1,25(OH)₂D₃ receptors (Fig. 1). Consequently, assay of TED-chromatin provides better quantitation (≥90% in intestinal mucosa) of the tissue content of unoccupied 1,25(OH)₂D₃ receptors, and hence, of the potential for physiological response to vitamin D, than do conventional cytosol assays (25).

The possible nuclear localization of unoccupied 1,25(OH)₂D₃ receptors and their solubilization (albeit incomplete) by STKM seemed at first to conflict with reconstitution experiments in which STKM-cytosol, chromatin, and [³H]-1,25(OH)₂D₃ were mixed and, subsequently, receptor-[³H]1,25(OH)₂D₃ complexes associated quantitatively with the chromatin (7, 19, 21, 22, 57). However, this discrepancy has been resolved: upon 1,25(OH)₂D₃ binding in vitro, a receptor activation (transformation) process occurs which results in a markedly higher affinity of the receptor 1,25(OH)₂D₃ complex for chromatin. Consequently, although STKM extracts a significant number of unoccupied receptors from chromatin (Figs. 4 and 6), it cannot extract occupied receptors. This differential effect of STKM probably results from its intermediate ionic strength (100 mM).

The observations reported here concerning the subcellular distribution of unoccupied 1,25(OH)₂D₃ receptors in vitro are consistent with reports from several other laboratories. In studies in rat intestinal mucosa (8, 9) and in mouse bone cells (16, 17) the inclusion of 0.3 M KCl in buffers proved necessary for detecting cytosol 1,25(OH)₂D₃ receptors. Additionally, the number of 1,25(OH)₂D₃ receptor detected in cytosol from the shell gland of rachitic chicks (58) improved markedly when KTED was used instead of STKM. Other reports of cytosolic 1,25(OH)₂D₃ receptors in multiple target sites (7–17) have employed either STKM or KTED for homogenization. In a preliminary series of experiments, Lawson and Wilson previously observed unoccupied nuclear binding sites for 1,25(OH)₂D₃ in vitro, even in purified nuclei (20). Importantly, due both to the increased receptor stability and to the simplicity of the Scatchard characteristics, the system described herein is more suitable for assessing the significance of these apparent nuclear binding sites.

The described distribution of the unoccupied 1,25(OH)₂D₃ receptors upon homogenization in TED could result from several phenomena: (a) nuclear or chromatin localization in vivo; (b) nuclear proximity in vivo leading to nuclear association in vitro; (c) ionic charge on the receptor molecule resulting in adsorption to oppositely charged ionic species in nucleoprotein upon nuclear damage in vitro. Thus the described receptors may not reflect the true in vivo localization of unoccupied 1,25(OH)₂D₃ receptors. However, all of these possible explanations would provide important information on either the physiological or biochemical properties of the 1,25(OH)₂D₃ receptor. Unfortunately, the extreme susceptibility of the receptor distribution patterns to ionic strength (Fig. 7) will make resolution of these mechanisms difficult. An additional complication is the possibility that under some conditions EDTA may enhance the nuclear association of these receptors. Thus, nuclear and chromatin purification techniques, which subject nuclei to ionic strength changes, osmotic effects, or EDTA, may give 1,25(OH)₂D₃ receptor distribution data which are virtually uninterpretable. Therefore, resolution of the actual mechanisms of the phenomena described herein will be accomplished most effectively when methods become available for observing the receptor molecule in situ in the absence of ligand.

Nevertheless, in several respects, the present results are reminiscent of the well established concept that unoccupied receptors for triiodothyronine (T₃) are located in nuclei and are indeed intrinsic non-histone chromosomal proteins (41, 42, 59). These T₃ receptors are located in nuclei and chromatin in low salt buffer and both occupied and unoccupied receptors can be extracted by buffer containing 0.4 M KCl (41, 42).

Importantly, De Groot and co-workers observed that STKM solubilizes the unoccupied T₃ receptors from nuclei (39). Additionally, the rebinding of these acidic T₃ receptors to chromatin is nonsaturable and is not tissue specific (41, 42). Similar properties have been observed for the 1,25(OH)₂D₃ receptors. Although it is presently unclear whether these similarities in receptor characteristics are physiologically meaningful, the parallels are certainly provocative.

In summary, in low salt buffers unoccupied 1,25(OH)₂D₃ receptors are associated with nuclear and chromatin fractions of target tissues. Whether this phenomenon represents a true nuclear localization of these unoccupied receptors in vivo or an unusually high affinity of cytoplasmic 1,25(OH)₂D₃ receptors for nuclei or chromatin in vitro has not been established. However, operationally, this characteristic presents many advantages. For example, 1,25(OH)₂D₃ receptor quantitation is best accomplished in TED-chromatin (25). Also, receptor visualization by sucrose density gradient ultracentrifugation and gel filtration chromatography is easier in chromatin extracts because of the virtual absence of the 25(OH)D₃-binding component. Additionally, competition analyses, where the vitamin D-binding protein also competes with the receptor for analog binding, can now be easily and unambiguously reassessed in the absence of this 25(OH)₁D₃ binding protein by the TED-chromatin assay. The absence of other cytoplasmic proteins renders the chromatin-associated receptors less susceptible to degradation. Perhaps most importantly, the increased receptor numbers and the purity of the chromatin preparation may help identify 1,25(OH)₂D₃ receptors in target tissues where they have been previously overlooked (ex. testes, Table II). Thus, regardless of the physiological significance of the nuclear association of unoccupied 1,25(OH)₂D₃ receptors, this attribute provides an important vehicle for further biochemical investigations.

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