Unoccupied 1,25-Dihydroxyvitamin D₃ Receptors

NUCLEAR/CYTOSOL RATIO DEPENDS ON IONIC STRENGTH

(Received for publication, January 31, 1980)

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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 was verified by their non-detection in nuclei of chicks treated with 200 units of 1,25(OH)₂D₃ 2 h prior to killing. The co-identity of these 1,25(OH)₂D₃ binding sites with "cytosol" receptors was confirmed by their extractability with high ionic strength buffers, by Scatchard analysis (Kₐ = 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, parathyroid, kidney, and pancreas; rat blast-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 [H]1,25(OH)₂D₃ administered in vitro or in vivo (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 20% w/v) with 10 strokes in a glass-Teflon homogenizer. After a low speed spin (5000 × g, 10 min) of the homogenate, cytosol was prepared by centrifuging at 105,000 × 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 × 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 × g, 10 min) and the supernatant (extract) was cleaned of debris (105,000 × g, 1 h).

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

* This work was supported by United States Public Health Service Grant AM-09012 and Training Grant AM-07310. This is Paper XXVII in a series, "Studies on the Mode of Action of Calciferol." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

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Unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} Receptors Associated with Nuclei

...alis in polypropylene test tubes (12 x 75 mm) with 5 nM 1,25(OH)\textsubscript{2}D, [26,27-\textsuperscript{3}H]D, (9 Ci/mmol) in the presence or absence of 200-fold excess unla... decreases the possibility of accidental pellet losses during the precipitation 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 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 in 5 ml of Amersham-Searle's ACS. Specific [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} binding was calculated by subtracting nonspecific binding ([\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} bound in the presence of excess 1,25(OH)\textsubscript{2}D\textsubscript{3} from total [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} binding.

Receptor Assay in Chick Kidney and Parathyroid Gland—The procedures outlined above for animal preparation and assay of unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} 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 maintained on the rachitogenic diet 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 containing 0.47% calcium, 1.2% phosphate, vitamin D-deficient diet (5, 27) for 7 to 8 weeks prior to decapitation. Small intestinal mucosa, kidney, and testes were removed and washed in 0.9% NaCl. All tissues were resuspended in TED + 10 mM Na\textsubscript{2}MoO\textsubscript{4} (33% w/v) prior to homogenization. Cytosol and crude chromatin were prepared as described above; chromatin was resuspended in TED/NaCl for incubation. Aliquots (200 µl) were incubated at 4°C for 2 h with 2 nM 1,25(OH)\textsubscript{2}D\textsubscript{3} (23,24-\textsuperscript{3}H]D\textsubscript{3}, 82 Ci/mmol ± 200-fold excess 1,25(OH)\textsubscript{2}D\textsubscript{3}; all tubes contained 50 nmoles radioactive 25(OH)D\textsubscript{3}. Specific receptor binding was assessed by the hydroxylapitate assay as described above.

Chemicals—1,25(OH)\textsubscript{2}D\textsubscript{3} [26,27-\textsuperscript{3}H]D\textsubscript{3} was prepared from Amersham-Searle's 9 Ci/mmol 25(OH)\textsubscript{2}D\textsubscript{3} [26,27-\textsuperscript{3}H]D\textsubscript{3}, by kidney homogenate hydroxylation (26). 1,25(OH)\textsubscript{2}D\textsubscript{3} (82 Ci/mmol) was obtained directly from Amersham-Searle, Arlington Heights, IL. Unlabeled 1,25(OH)\textsubscript{2}D\textsubscript{3} and 25(OH)D\textsubscript{3} were the kind gifts of Hoffmann-LaRoche (Nutley, NJ). Hydroxylapitate was purchased from Bio-Rad, Richmond, CA. Triton X-100 was obtained from Sigma Chemical Co., St. Louis, MO. The 8-200 gel for gel filtration chromatography was purchased from Pharmacia, Piscataway, NJ.

RESULTS

Cytosol and Nuclear Distribution of Mucosal 1,25(OH)\textsubscript{2}D\textsubscript{3} Receptors in TED and KTED—As a basis for subsequent comparisons, Table I summarizes the amounts of 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors detected in various cytosol preparations from the intestinal mucosa of vitamin D-deficient chicks. Very little, 1,25(OH)\textsubscript{2}D\textsubscript{3} 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 KTED 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)\textsubscript{2}D\textsubscript{3} 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 as described above, and receptors were assessed by incubating with [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} as described under "Materials and Methods." Surprisingly, a large number of unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)D, with high affinity, is prominent in plasma and is also present in many cytosol preparations (31–34). When [\textsuperscript{3}H]25(OH)D\textsubscript{3} binding was measured in mucosal cytosol and chromatin prepared in TED, only 12% of the specific 25(OH)\textsubscript{2}D\textsubscript{3} binding was observed in the chromatin fraction (Fig. 1B). These data suggested that the nuclear association of the unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors results from a specific biochemical property of the receptor molecules.

Unoccupied Nature of the Nuclear 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} 2 h prior to killing. TED-chromatin was prepared and was incubated with 5 nM [\textsuperscript{3}H]25(OH)\textsubscript{2}D\textsubscript{3} ± 1 µmol unlabeled 25(OH)\textsubscript{2}D\textsubscript{3} at 4°C for 2 or 24 h. As shown in Fig. 2, very little [\textsuperscript{3}H]25(OH)\textsubscript{2}D\textsubscript{3} exchanged with the endogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} bound to receptors in injected chicks under these incubation conditions, confirming the unfilled nature of the 1,25(OH)\textsubscript{2}D\textsubscript{3} 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 [\textsuperscript{3}H]25(OH)\textsubscript{2}D\textsubscript{3} binding is not simply a non-receptor artifact of the in vitro chromatin preparation.

Characteristics of Residual Nuclear Unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} Receptors—The chromatin-associated [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} binding sites could represent a non-receptor moiety which was nevertheless inhibitable by excess 1,25(OH)\textsubscript{2}D\textsubscript{3}. Thus, we tested whether these sites could be extracted from the chromatin and still retain their [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} binding capability. Cytosol and chromatin from the intestinal mucosa of vitamin D-deficient chicks were prepared in KTED or STKM; then the chromatin preparations were re-extracted with TED and KTED plus 0.5% Triton X-100, and the above described methods were repeated. The results of these experiments are shown in Table II. The specific 25(OH)\textsubscript{2}D\textsubscript{3} binding was not present in the re-extracted cytosol and chromatin.

Table I

Relative amounts of 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors in cytosol prepared in different buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Relative specific binding</th>
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<tr>
<td>KTED</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>STKM</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>TEDG</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>TED</td>
<td>0.14 ± 0.06</td>
</tr>
</tbody>
</table>
The homogeneity of the unoccupied 1,25(OH)$_2$D$_3$ receptors in the chromatin was assessed further by Scatchard analysis (35) of their co-identity with the 1,25(OH)$_2$D$_3$ receptors of the intestinal mucosa. Rather, the data of Fig. 4 provide further evidence for their co-identity with the 1,25(OH)$_2$D$_3$ receptors of the intestinal mucosa.

The homogeneity of the unoccupied receptors in the chromatin was explored by gel filtration chromatography. STKM extracts of TED-chromatin or KTED extracts of TED-chromatin were incubated with 5 nM [H]$^3$1,25(OH)$_2$D$_3$ at 4°C for 2 h. Then 500 µl aliquots of the extracts or other tissue preparations were applied to an S200 (Pharmacia) column (0.5 x 85 cm) in the cold room. Samples were eluted with the equilibrium buffer (KTED) at a flow rate of 8 ml/h. Importantly, the [H]$^3$1,25(OH)$_2$D$_3$ elution patterns in the chromatin extracts were similar to those of the control cytosol preparations (Fig. 4). To confirm that the [H]$^3$1,25(OH)$_2$D$_3$ binding in these chromatin extracts was not due to nonreceptor [H]$^3$25(OH)$_2$D$_3$ binding components, preparations of cytosol and chromatin extracts were incubated with [H]$^3$25(OH)$_2$D$_3$ and then were applied to the S200 column. As expected, cytosol contained a substantial [H]$^3$25(OH)$_2$D$_3$ binding component (Fig. 5), which eluted in an area of the hydroxylapatite assay. An additional 30 to 40% specific [H]$^3$1,25(OH)$_2$D$_3$ binding sites could be solubilized from the chromatin by KTED after cytosol preparation in either STKM (Fig. 3) or KTED (not shown). In further experiments, KTED extraction was used as above usually solubilized 50 to 75% of the residual chromatin receptor sites. The variation in this percentage probably reflects inefficiency in solubilization rather than the presence of a distinct nonextractable moiety (see below).

The identity of the 1,25(OH)$_2$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 [H]$^3$1,25(OH)$_2$D$_3$ at 4°C for 2 h. Then 500 µl aliquots of the extracts or other tissue preparations were applied to an S200 (Pharmacia) column (0.5 x 85 cm) in the cold room. Samples were eluted with the equilibrium buffer (KTED) at a flow rate of 8 ml/h. Importantly, the [H]$^3$1,25(OH)$_2$D$_3$ elution patterns in the chromatin extracts were similar to those of the control cytosol preparations (Fig. 4). To confirm that the [H]$^3$1,25(OH)$_2$D$_3$ binding in these chromatin extracts was not due to nonreceptor [H]$^3$25(OH)$_2$D$_3$ binding components, preparations of cytosol and chromatin extracts were incubated with [H]$^3$25(OH)$_2$D$_3$ and then were applied to the S200 column. As expected, cytosol contained a substantial [H]$^3$25(OH)$_2$D$_3$ binding component (Fig. 5), which eluted in an area of the column similar to the 1,25(OH)$_2$D$_3$ receptors (compare Figs. 3 and 4). However, no [H]$^3$25(OH)$_2$D$_3$ binding was observed in the chromatin extracts (Fig. 5). This result and the data of Fig. 4B confirm that high affinity 25(OH)$_2$D$_3$ binding proteins are not the source of the chromatin binding sites observed herein. Rather, the data of Fig. 4 provide further evidence for their co-identity with the 1,25(OH)$_2$D$_3$ receptors of the intestinal mucosa.

The homogeneity of the unoccupied receptors in the chromatin was assessed further by Scatchard analysis (35) of various subcellular fractions of intestinal mucosa of vitamin D$_3$-deficient chicks. Aliquots of these subcellular fractions were incubated with 0.2 to 10 nM [H]$^3$1,25(OH)$_2$D$_3$ ± 1 µM unlabeled 1,25(OH)$_2$D$_3$ at 4°C for 18 to 24 h, a time sufficient to achieve equilibrium (25). Single component linear Scatchard plots with $K_d = 0.65$ to 2.2 nM were obtained in all tissue preparations (Fig. 6): STKM-cytosol (A), KTED-cytosol (B), TED-cytosol (C), TED-chromatin (D), STKM extract of TED-chromatin (E), and KTED extract of TED-chromatin (F). These data are consistent with the hypothesis that the 1,25(OH)$_2$D$_3$ binding sites in all these preparations represent a single population of classical hormone receptors.

Effect of Buffer Ionic Content on Subcellular Distributions of the Unoccupied 1,25(OH)$_2$D$_3$ Receptors—The difference in the distribution of unoccupied 1,25(OH)$_2$D$_3$ receptors in KTED as opposed to TED can be readily explained by the differences in their ionic concentration. Indeed, 0.3 to 0.4 M KCl can extract many intrinsic nuclear components (36-38), and sucrose which may preserve nuclear integrity (43), thus also preserving the true in vivo receptor distribution. In separate experiments mucosal preparations of vitamin D$_3$-deficient chicks were mixed in TED or in TKM prior to complete homogenization of aliquots in TED, TED ± 5 mM MgCl$_2$, and KTED or in STKM and TKM, respectively. The addition of 5 mM MgCl$_2$ 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)$_2$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 70% 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 or in the presence of 10 nM [3H]25(OH)2D3 ± excess 1,25(OH)2D3. Then 500 µl of hydroxylapatite and 1 mL of TED Triton were added and the
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 in Target Tissues of Chicks and Rats—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.

TABLE II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1,25(OH)$<em>{2}$D$</em>{3}$ Receptors</th>
<th>(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Chromatin</td>
</tr>
<tr>
<td>Chick</td>
<td>9.9 ± 1.6</td>
<td>90.1 ± 1.6</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>18</td>
<td>82</td>
</tr>
<tr>
<td>Parathyroid gland</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>Kidney</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>Pancreas$^{c}$</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>Rat</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>Bone cell culture</td>
<td>16</td>
<td>84</td>
</tr>
</tbody>
</table>

$^{a}$ Data from S. Christakos and A. W. Norman (from this laboratory).

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.$^{7}$

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

$^{7}$ W. Hunziker, M. R. Walters, and A. W. Norman, unpublished observations.
(25 to 50%) of the tissue unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors (Fig. 1). Consequently, assay of TED-chromatin provides better quantitation (≥20% in intestinal mucosa) of the tissue content of unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} receptors and their solubilization (albeit incomplete) by STKM seemed at first to conflict with reconstitution experiments in which STKM-cytosol, chromatin, and [\textsuperscript{3}H]-1,25(OH)\textsubscript{2}D\textsubscript{3} were mixed and, subsequently, receptor-\[\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} complexes associated quantitatively with the chromatin (7, 19, 21, 22, 57). However, this discrepancy has been resolved: upon 1,25(OH)\textsubscript{2}D\textsubscript{3} binding in vitro, a receptor activation (transformation) process occurs which results in a markedly higher affinity of the receptor 1,25(OH)\textsubscript{2}D\textsubscript{3} complex for chromatin.\textsuperscript{2} Consequently, although STKM extracts a significant number of unoccupied receptors from chromatin (Figs. 4 and 6), it cannot extract occupied receptors.\textsuperscript{2} 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)\textsubscript{2}D\textsubscript{3} 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 KC\textsubscript{1} in buffers proved necessary for detecting cytosolic 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors. Additionally, the number of 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors detected in cytosol from the cell gland of rachitic chicks (58) improved markedly when KTED was used instead of STKM.\textsuperscript{3} Other reports of cytosolic 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors in multiple target tissues (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)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} 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 charges on the receptor molecule resulting in adsorption to oppositely charged ionic species in nuclei, phenomenon upon nuclear damage in vitro. Thus the described receptors may not reflect the true in vivo localization of unoccupied 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors. However, all of these possible explanations would provide important information on either the physiological or biochemical properties of the 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} 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 (T\textsubscript{3}) are located in nuclei and are indeed intrinsic non-histone chromosomal proteins (41, 42, 59). These T\textsubscript{3} 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 KC\textsubscript{1} (41, 42). Importantly, De Groot and co-workers observed that STKM solubilizes the unoccupied T\textsubscript{3} receptors from nuclei (39). Additionally, the rebinding of these acidic T\textsubscript{3} receptors to chromatin is nonsaturable and is not tissue specific (41, 42). Similar properties have been observed for the 1,25(OH)\textsubscript{2}D\textsubscript{3} receptors.\textsuperscript{2} 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)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} receptors for nuclei or chromatin in vitro has not been established. However, operationally this characteristic presents many advantages. For example, 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3}-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)\textsubscript{2}D\textsubscript{3} binding protein by the TED-chromatin assay. The absence of other cytoplasmic proteins renders the chromatin-associated receptors less susceptible to degradation.\textsuperscript{2} Perhaps most importantly, the increased receptor numbers and the purity of the chromatin preparation may help identify 1,25(OH)\textsubscript{2}D\textsubscript{3} 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)\textsubscript{2}D\textsubscript{3} receptors, this attribute provides an important vehicle for further biochemical investigations.

REFERENCES

\textsuperscript{2} W. A. Coty, personal communication.