Biochemical Pharmacology, Vol. 33, No. 7, pp. 1047–1057, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

INHIBITORY ACTION OF AURINTRICARBOXYLIC ACID AND RIFAMYCIN AF/013 AT THE POLYNUCLEOTIDE DOMAIN OF 1,25-DIHYDROXYVITAMIN D_3-RECEPTOR COMPLEXES*

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(*Received* 3 *February* 1983; *accepted* 26 *August* 1983)

Abstract—The binding of 1,25-dihydroxyvitamin D_3-receptor complexes from chicken intestine to DNA-cellulose and isolated intestinal nuclei is inhibited in a dose-dependent manner by aurintricarboxylic acid and rifamycin AF/013. Since both nuclear- and cytoplasmic-associated receptors have been identified, some experiments were carried out on both populations of receptors. Concentrations resulting in 50% displacement of cytoplasmic receptor complexes were 3.2×10^{-6} M and 1.2×10^{-4} M for aurintricarboxylic acid and rifamycin AF/013 respectively. Moreover, rifamycin AF/013 was approximately nine times more potent at inhibiting nuclear receptor binding to DNA-cellulose compared to cytoplasmic receptors. Contrary to these findings, rifampicin, which does not inhibit eukaryotic RNA or DNA polymerases, did not cause a loss of receptor complex binding to DNA-cellulose at the doses tested. Neither aurintricarboxylic acid, rifampicin, nor rifamycin AF/013 resulted in any significant loss of sterol binding. Inhibition of receptor binding to DNA-cellulose by these polymerase inhibitors was not due to alteration of the DNA and was reversed by dialysis. Incubation of receptor complexes with aurintricarboxylic acid or rifamycin AF/013 inhibited binding to Cibacron blue-agarose and phosphocellulose. Furthermore, these polymerase inhibitors were utilized specifically to desorb receptor complexes from Cibacron blue-agarose columns. Sucrose density gradient analysis of inhibitor treated and untreated receptor revealed that rifamycin AF/013 treatment resulted in the appearance of a broadened 3.7 S sedimenting receptor in addition to specific bound 1,25-dihydroxyvitamin D_3 in the 6.0 S region and in the pellet of the gradient.

The precise mechanism whereby 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) elicits physiological and pharmacological responses in target cells is not completely understood. Although high affinity, low capacity cytoplasmic receptors in various target tissues have been implicated in $1,25(OH)_2D_3$ action (see Ref. 1 for a review), there is no direct evidence that they mediate the actions of the hormone. The most studied target tissue employed to elucidate the mechanism of $1,25(OH)_2D_3$ action is the intestine. Utilizing this tissue, evidence has been accumulated which implicates $1,25(OH)_2D_3$–receptor complexes in the physiological and pharmacological responses of intestine to $1,25(OH)_2D_3$. Both autoradiographic [2] and subcellular fractionation studies [3–5] have demonstrated that $1,25(OH)_2D_3$ accumulates in the nucleus *in vivo* with a time course consistent with the nuclear binding being a prerequisite for hormone-dependent calcium transport. In addition, analysis of total intestinal mucosal radioactivity revealed that the majority of it is located in the chromatin fraction [6, 7]. In concert with these findings, chromatin isolated from intestinal mucosa of chicks treated with radioactive $1,25(OH)_2D_3$ contains much of the sterol bound to salt extractable receptors [5, 8]. A portion of the nuclear bound $1,25(OH)_2D_3$ is released by DNAse treatment [5], indicating that some of the sterol is bound to DNA or a DNA-associated nuclear component.

It seems plausible that $1,25(OH)_3D_3$ receptors account for much of the nuclear accumulation of $1,25(OH)_2D_3$. However, it is not clear if cytoplasmic receptors mediate the translocation of $1,25(OH)_2D_3$ into the nucleus. Studies have shown that unoccupied receptors prepared under conditions of low ionic strength remain associated with the crude nuclear pellet [9]. Finally, direct evidence of receptor involvement in $1,25(OH)_2D_3$ action has been provided by Zerwekh *et al.* [10]. Chromatin from nuclei was reconstituted with $1,25(OH)_2D_3$-labeled cytosol, which resulted in an increase in total RNA synthesis. This response required both cytosol and $1,25(OH)_2D_3$. Taken collectively, these findings provide support for the hypothesis that $1,25(OH)_2D_3$ receptors mediate the hormonal action of $1,25(OH)_2D_3$.

Recently, $1,25(OH)_2D_3$–receptor complexes have been shown to bind selectively *in vitro* to synthetic deoxynucleotide polymers [11]. Similar findings have been demonstrated for DNA polymerase-α [12] and estradiol receptor complexes [13, 14]. The polymerase inhibitors aurintricarboxylic acid (ATA) and rifa-

* This work was supported in part by NIH Program Project Grant AM-27234 and the Graduate School of the University of Wisconsin-Madison.

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mycin AF/013 have been shown to interact with both progesterone and estradiol receptors, which results in inhibition of binding to synthetic deoxynucleotides or nuclei [15–17]. Moreover, polymerase inhibitors and anti-DNA polymerase-α IgG have been utilized to probe for similarities between the cytoplasmic rat hepatic glucocorticoid receptors and DNA polymerase-α [18]. These observations suggest that steroid hormone receptors and polymerases, as DNA-binding proteins, may have similar polynucleotide sites. The present study was undertaken to investigate whether the known polymerase inhibitors ATA and rifamycin AF/013 inhibit $1,25(OH)_2D_3$–receptor complex DNA binding. Such a finding would strengthen the possibility that these receptor molecules have or are associated with polymerase activity.

MATERIALS AND METHODS

Animals. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) were fed a vitamin D-deficient soy protein diet (Teklad Test diet, TD 80146, Madison, WI) and received water *ad lib.* for 3–4 weeks. All birds were maintained in a vivarium at 24–26° on an alternating 12-hr light-and-dark cycle using incandescent lighting.

Chemicals. Nonradioactive $1,25(OH)_2D_3$ was a gift from Dr. M. Uskokovic of the Hoffmann-LaRoche Co. (Nutley, NJ). Determination of purity and concentration of nonradioactive $1,25(OH)_2D_3$ was achieved by ultraviolet absorption spectroscopy using an extinction coefficient ($\varepsilon = 264$) of $18,200\ M^{-1}\ cm^{-1}$. $1,25(OH)_2[26,27\text{-}^3H]D_3$ (148–163 Ci/mmole) was obtained from the Amersham Corp. (Arlington Heights, IL). Rifamycin AF/013 (*O-n*-octyloxime of 3-formyl rifamycin SV) was provided by Dr. Giancarlo Lancini of the Gruppo Lepetit (Milano, Italy). Other chemicals used and their sources were: blue A matrix (Cibacron blue F3GA bound to agarose via an ether linkage through the triazine ring), Amicon Corp. (Lexington, MA); cellulose (Cellex 410) and hydroxylapatite (HAP) (bioGel HTP), BioRad Laboratories (Richmond, CA); sucrose (ultrapure), Schwarz/Mann (Orangeburg, NY); dimethyl sulfoxide (DMSO) (certified) and ethylene glycol (certified), Fisher Scientific (Fair Lawn, NJ); diethylaminoethyl-cellulose (DEAE-cellulose) (DE-52) and phosphocellulose (P-11), Whatman, Inc. (Clifton, NJ); 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazole)]benzene (POPOP), and Triton X-100, RPI Corp. (Elk Grove Village, IL); and dialysis sacks (10 in. × 0.62 in.), calf thymus DNA, charcoal (neutralized and washed to remove the "fines"), dextran (M_r 70,000), Tris, bovine serum albumin (fraction V), EDTA, dithiothreitol (DTT), rifampicin (3-[4-methylpiperazinyliminomethyl] rifamycin SV), and ATA (ammonium salt), Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Preparation of cytoplasmic $1,25(OH)_2D_3$ *receptors.* Chickens were deprived of food for 16–20 hr after which time they were killed by cervical dislocation and the duodenal loop was free of pancreas and excised. All procedures were carried out between 0 and 4°. Mucosa was scraped free of serosa and washed three times in several volumes (w/v) of buffer, 50 mM Tris–HCl, 1.5 mM EDTA, 0.5 mM DTT, and 0.15 M KCl, pH 7.5 (TEDK-0.15). Washed mucosa was homogenized in 5 vol. of TMDK-0.30 buffer (except, the concentrations of $MgCl_2$ and DTT were 5.0 mM) as described previously [11]. The cytoplasmic fraction, containing $1,25(OH)_2D_3$ binding proteins, was prepared by centrifuging the homogenate at 78,000 *g* for 90 min in a Beckman L5-50B ultracentrifuge using a type 30 rotor (Beckman Instruments, Palo Alto, CA). Cytosol was frozen in an isopropanol/dry ice bath, followed by lyophilization (FTS Systems Inc., Stone Ridge, NY), and stored at −70° until use.

Isolation of chicken intestinal nuclei and preparation of nuclear $1,25(OH)_2D_3$ *receptors.* Nuclei were isolated by the hexylene glycol procedure described by Wray *et al.* [19]. Intestinal mucosa was homogenized at 0–4° in hexylene glycol buffer (0.5 M hexylene glycol, 5 mM Tris–HCl, 0.1 mM $CaCl_2$, and 0.5 mM DTT, pH 7.5) in a Dounce homogenizer (10–12 passes). The homogenate was filtered through four layers of cheesecloth and then through a single layer of monofilament nylon screen (Nitex 102 μm) (Tetko, Inc., Elmsford, NY) followed by centrifugation for 5 min at 900 *g* in a Beckman TJ-6R centrifuge employing a TH4 rotor. This procedure was repeated two additional times. The washed nuclear pellet was then suspended in sucrose buffer (2.0 M sucrose, 10 mM Tris–HCl, and 0.1 mM $MgCl_2$, pH 7.5) and centrifuged at 20,000 *g* for 45 min in a Beckman ultracentrifuge L5-50B using a type 30 rotor. The supernatant fraction was removed, and the nuclear pellet was diluted in TMDK-0.03 buffer. The DNA content of aliquots of the nuclear suspension was quantitated by the method of Richards [20].

To prepare nuclear $1,25(OH)_2D_3$ receptors, the nuclei suspended in TMDK-0.03 buffer were pelleted, and the supernatant fraction was aspirated. The nuclei were extracted with TMDK-0.6 buffer at 0–4° for 45 min with frequent blending on a vortex mixer. The nuclear chromatin extract was diluted with TMD buffer to make a final KCl concentration of 0.3 M and then centrifuged at 105,000 *g* in a Ti 50 rotor (Beckman Instruments) for 45 min. The clear supernatant fraction was utilized for the source of $1,25(OH)_2D_3$ receptors. Nuclear and cytoplasmic protein concentrations were determined by the method of Lowry *et al.* [21] using crystalline bovine serum albumin as a standard.

Receptor labeling and quantitation. Receptor preparations were incubated with 2.0 nM $1,25(OH)_2[26,27\text{-}^3H]D_3$ (148–163 Ci/mmole) at 0–4° in the presence (nonspecific binding) or absence (total binding) of a 200-fold excess of nonradioactive $1,25(OH)_2D_3$ for 3.5 hr or 4.5 hr for cytosol and nuclear extracts respectively. Radioactive and nonradioactive sterol were added in cold absolute ethanol (2.5 to 5.0%, v/v). After saturation, the labeled receptor preparations were incubated further with an inhibitor or the appropriate vehicle. ATA was dissolved in TMDK-0.03 buffer, while stock solutions of rifampicin and rifamycin AF/013 were prepared by dissolving the compounds in TMDK-0.03 containing 10% DMSO (v/v). These solutions were further diluted with TMDK-0.03 buffer so that the final concentration of

DMSO in the reaction mixture was less than 0.1% (v/v). All inhibitors were incubated with $1,25(OH)_2[^3H]D_3$-receptor complexes for 1.0 hr at 0–4° (this time results in maximal inhibition of DNA binding). Subsequently, the unbound sterol was removed by dextran-coated charcoal adsorption [0.05% and 0.5%, (w/v), respectively]. The $1,25(OH)_2[^3H]D_3$–receptor complexes were diluted with TMD buffer to lower the KCl concentration to an appropriate level (specified in the figure legends)

Bound sterol was quantitated by an HAP batch assay [22]. Briefly, 0.25 to 0.50 ml of HAP slurry [50% (v/v) in 50 mM Tris–HCl, 0.1 M KCl buffer, pH 7.5] was added to each incubation tube containing $1,25(OH)_2[^3H]D_3$–labeled cytosol or nuclear extract (0.25 to 0.50 ml) in the presence of inhibitors or an equal volume of vehicle. The samples were kept at 0–4° for 15 min with intermittent mixing and centrifuged at 1500 *g* for 5 min. The HAP pellet was washed three times with 1.0 ml of 10 mM Tris, 0.5 mM DTT, and 0.1% Triton X-100 (v/v) buffer, pH 7.5. The washed HAP pellets were extracted two times with 1.0 ml portions of chloroform–methanol (1:2, v/v); the supernatant fractions were combined and dried under a stream of air at 35°. To each vial was added 4.0 ml of a scintillation fluid consisting of 1.32 liters of Triton X-100, 16 g PPO, and 0.2 g POPOP per 4 liters of toluene. The radioactivity was quantitated by liquid scintillation spectrometry in a Packard Tri-Carb Spectrometer (model Prias PLD) with an efficiency for tritium of 45%. Quench correction was determined by the use of automatic external standardization.

Receptor binding to DNA-cellulose. The preparation of DNA-cellulose was performed as described previously [23]. Quantitation of $1,25(OH)_2[^3H]D_3$–receptor complex binding to DNA-cellulose in TMDK-0.03 buffer was conducted by the method of Radparvar and Mellon [11]. For the binding assay, aliquots of DNA-cellulose suspensions (containing 50 μg calf thymus DNA/aliquot, unless otherwise stated) (total binding) or equal volumes of cellulose suspension (nonspecific binding) were placed into glass scintillation vials and washed with TMD buffer. The reaction mixtures (0.5 to 0.84 ml/incubation) consisting of $1,25(OH)_2[^3H]D_3$–receptor complexes (0.03 to 0.08 nM), Tris (50 mM), $MgCl_2$ (0.5 mM), DTT (0.5 mM), and KCl (0.03 M), pH 7.5, were incubated with DNA-cellulose or cellulose pellets in the presence of inhibitors or an equal volume of vehicle and quantitated as previously described [11]. In some experiments, isolated intestinal nuclei were utilized instead of DNA-cellulose in the reaction mixtures described above.

Sucrose density gradient analysis. Cytoplasmic and nuclear receptor preparations were labeled with 2.0 nM $1,25(OH)_2[^3H]D_3$ in the presence or absence of a 200-fold excess of nonradioactive sterol at 0–4° as described above. After saturation with sterol, portions of the receptor were treated with ATA, rifamycin AF/013, or the appropriate vehicle previously described. The labeled receptor was subjected to dextran-coated charcoal treatment. Aliquots of the supernatant fraction were applied to linear sucrose density gradients (4–20%) containing an appropriate concentration of inhibitor to observe sedimentation characteristics of the sterol–receptor complexes. Gradients were centrifuged using a Beckman SW-60 rotor and subsequently fractionated as previously described by Kream *et al.* [24]. To each 0.1-ml fraction was added 4.0 ml of a scintillation fluid mixture consisting of 1.32 liters of Triton X-100, 16 g PPO, 0.2 g POPOP per 4.0 liters of toluene, and sufficient water to effect clarification. The radioactivity was determined by liquid scintillation spectrometry as described above.

Statistical analysis. Where appropriate, statistical comparisons of the independent samples means were made using Student's *t*-test at the 95% level of confidence.

RESULTS

To assess the relative potencies of several polymerase inhibitors with respect to their abilities to compete with DNA for the polynucleotide domain of $1,25(OH)_2D_3$–receptor complexes, DNA-cellulose was incubated in the presence, or absence, of several concentrations of inhibitors along with $1,25(OH)_2[^3H]D_3$–receptor complexes (Fig. 1). Comparison of inhibitors was evaluated at the concentration of competitor which resulted in 50% loss of specific DNA binding. Due to the existence of cytoplasmic- and nuclear-associated receptors, some studies were conducted to compare both populations of receptors. The concentration of ATA causing 50% displacement of cytoplasmic $1,25(OH)_2D_3$–receptor complexes was 3.2×10^{-6} M (Fig. 1). The dose at which rifamycin AF/013 inhibited 50% of the DNA-cellulose binding differed for $1,25(OH)_2D_3$ receptors isolated from cytosol and nuclei (1.2×10^{-4} and 1.4×10^{-5} M respectively). However, rifampicin, at the doses tested (5×10^{-5} to 5×10^{-4} M), did not inhibit receptor complex binding to DNA-cellulose.

To ensure that the apparent displacement of receptor binding to DNA-cellulose was not due to loss of hormone binding, concomitant binding assays monitoring sterol binding by the HAP procedure were carried out. For several concentrations, neither ATA, rifampicin, nor rifamycin AF/013 resulted in any significant losses of $1,25(OH)_2D_3$ (Fig. 1). Moreover, an experiment was conducted to ascertain whether the inhibitory action of ATA and rifamycin AF/013 occurred by binding to DNA-cellulose or to the $1,25(OH)_2D_3$–receptor complex. DNA-cellulose pellets were untreated or incubated with ATA (2.5×10^{-5} M) or rifamycin AF/013 (2×10^{-4} M) for 30 min at 0–4°. The untreated and a portion of the inhibitor-treated DNA-cellulose were washed three times with TMDK-0.03 buffer. Subsequently, $1,25(OH)_2[^3H]D_3$–receptor complexes were incubated with the DNA-cellulose suspensions. In those vials containing inhibitors and DNA-cellulose, there was a significant reduction in $1,25(OH)_2D_3$–receptor complex binding, 95% and 97% for ATA and rifamycin AF/013 respectively (Table 1). When the inhibitor-treated DNA-cellulose was washed with buffer prior to receptor complex addition, there was no loss of binding to DNA-cellulose compared to the control (DNA-cellulose pellets washed with buffer only).

Similar findings in the presence of polymerase inhibitors were observed when nuclei were substi-

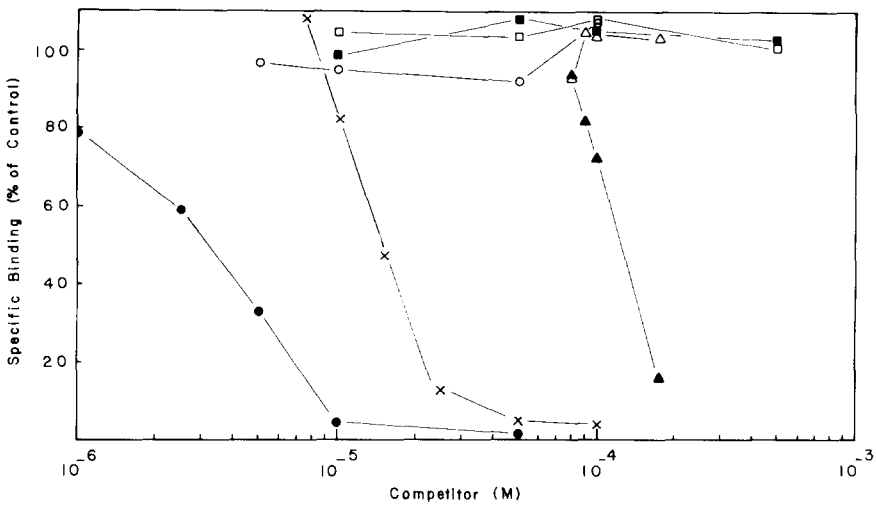

Fig. 1. Effects of several inhibitors on 1,25(OH)$_2$[^{3}H]D$_3$ binding to chick intestinal receptors and on receptor complex binding to DNA-cellulose. Chick intestinal cytosol (5.0 to 9.5 mg protein/ml) or nuclear extract (0.57 mg protein/ml) was incubated with 2.0 nM 1,25(OH)$_2$[26,27-^{3}H]D$_3$ (148–160 Ci/mmole) in the presence or absence of a 200-fold excess of nonradioactive 1,25(OH)$_2$D$_3$ as described in Materials and Methods. After the incubation period, various concentrations of inhibitors were added to the reaction mixtures. Subsequently, unbound sterol was removed by dextran-coated charcoal. Sterol binding (open symbols) and DNA binding (closed symbols) were assessed by the HAP procedure and DNA-cellulose, respectively, for ATA (○, ●), rifamycin AF/013 (△, ▲), and rifampicin (□, ■). Rifamycin AF/013 inhibition of nuclear 1,25(OH)$_2$[^{3}H]D$_3$–receptor complex binding to DNA-cellulose is represented by the symbol (×). Specific binding was quantitated by subtracting the nonspecific binding from the total binding values. The quantity of receptor binding in the presence of inhibitors is presented as the percent specific binding compared to control (vehicle-treated receptors). The amount of DNA-cellulose binding for control receptor incubations for these experiments ranged between 71 and 76% of specific receptor complexes (quantitated by the HAP procedure) added to the reaction mixtures. Values represent the mean for three to four determinations.

tuted for DNA-cellulose. After addition of 1,25(OH)$_2$[^{3}H]D$_3$–receptor complexes to chicken intestinal nuclei in the presence of various concentrations of ATA or rifamycin AF/013, there was a dose-dependent reduction of receptor binding (Table 2). Similar to the results obtained using DNA-cellulose, rifampicin was unable to inhibit significantly receptor binding to nuclei at the doses employed.

The inhibitory action of ATA and rifamycin AF/013 on 1,25(OH)$_2$D$_3$–receptor complex binding to DNA-cellulose can be reversed by dialysis. Data presented in Table 3 demonstrate that labeled cytosol, incubated in the presence of ATA (2.5×10^{-5} M) or rifamycin AF/013 (2×10^{-4} M) and then subjected to dialysis for 3.0 hr at 0–4°, restored DNA-cellulose binding to 88 and 109% of similarly dialyzed control cytosol respectively. Whereas ATA and rifamycin AF/013 caused significant inhibition of DNA-cellulose binding, there was no significant loss of sterol binding in the presence or absence of dialysis.

To characterize further the inhibitory action of

Table 1. Effects of ATA and rifamycin AF/013 on DNA-cellulose integrity*

Treatment of DNA-cellulose	Specific pg 1,25(OH)$_2$[^{3}H]D$_3$ bound/DNA-cellulose pellet†
TMDK-0.03 washes only	7.00 ± 0.79
ATA, 2.5×10^{-5} M	0.32 ± 0.21‡
ATA, 2.5×10^{-5} M, followed by 3× TMDK-0.03 washes prior to receptor addition	8.66 ± 0.94
Rifamycin AF/013, 2×10^{-4} M	0.18 ± 0.11‡
Rifamycin AF/013, 2×10^{-4} M, followed by 3× TMDK-0.03 washes prior to receptor addition	7.18 ± 0.67

* Cytosol was incubated with 2.0 nM 1,25(OH)$_2$[26,27-^{3}H]D$_3$ (158 Ci/mmole) as described in Materials and Methods. Receptor complexes were added to DNA-cellulose pellets which had been washed with buffer only, treated with an inhibitor, or treated with an inhibitor followed by buffer washes.

† Values represent the mean ± S.D. of triplicate determinations.

‡ Statistically significant from DNA-cellulose pellets washed with TMDK-0.03 only, $P < 0.05$.

Table 2. Effects of ATA, rifampicin and rifamycin AF/013 on $1,25(OH)_2[^3H]D_3$–receptor complex binding to isolated chick intestinal nuclei*

Compound	Concentration (M)	Specific $1,25(OH)_2[^3H]D_3$–receptor complexes bound/nuclear pellet (% of control)†
ATA	7.5×10^{-6}	54.0 ± 3.4‡
	2.5×10^{-5}	15.3 ± 1.2‡
	5.0×10^{-5}	1.7 ± 0.3‡
Rifampicin	1.0×10^{-4}	110.9 ± 12.1
	5.0×10^{-4}	90.3 ± 17.6
Rifamycin AF/013	1.0×10^{-4}	68.6 ± 10.2‡
	2.0×10^{-4}	7.5 ± 6.2‡

* Chicken intestinal cytosol (4.2 mg protein/ml) was incubated with 2.0 nM $1,25(OH)_2$-[26,27-^{3}H]D_3 (158 Ci/mmole) as described in Materials and Methods. For the binding assay, receptor complexes were added to the reaction mixtures containing aliquots of suspended nuclei (containing 125–160 μg DNA) in TMDK-0.03 buffer.

† Values represent the mean ± S.D. for triplicate determination expressed as percent of control. Control cytosol (no inhibitor) had values of 7.1 to 9.4 pg of specific $1,25(OH)_2[^3H]D_3$ bound per nuclear pellet.

‡ Significantly different from control (100%) value, $P < 0.05$.

ATA and rifamycin AF/013 with respect to DNA binding and to verify that this effect was mediated through a common site on the protein, the inhibitory data were analyzed by double-reciprocal plots. When a constant amount of $1,25(OH)_2[^3H]D_3$–receptor complexes was added to increasing concentrations of DNA-cellulose (1.0 to 5.0 μg DNA) in the absence or presence of ATA (2×10^{-6} M) or rifamycin AF/013 (1.75×10^{-5} M), the reciprocal plots of the binding data revealed typical competitive inhibition patterns (Fig. 2).

Several chromatographic media were employed to further delineate the interaction of ATA and rifamycin AF/013 with $1,25(OH)_2D_3$–receptor complexes. Only the chromatographic profiles for either ATA or rifamycin AF/013 are presented to avoid duplicate figures. Where deviations occur, they are stated in the text. $1,25(OH)_2D_3$–receptor complexes formed at 0–4° were incubated in the presence or absence (control) of ATA (10^{-4} M) and applied to a Cibacron blue-agarose column. Control cytosol bound to the matrix and was eluted predominately at a KCl concentration of 0.8 M (Fig. 3A). In contrast, the portion of the labeled cytosol that was incubated with ATA appeared mainly in the drop-through fractions (Fig. 3B). Since rifamycin AF/013 (data not shown) and ATA (Fig. 3B) could inhibit receptor binding to Cibacron blue-agarose, these inhibitors were tested for their abilities to elute $1,25(OH)_2D_3$–receptor complexes from the dye-

Table 3. Reversal of ATA and rifamycin AF/013 inhibition of $1,25(OH)_2D_3$–receptor complex binding to DNA-cellulose by dialysis*

Treatment of $1,25(OH)_2[^3H]D_3$–receptor complexes	Specific pg $1,25(OH)_2[^3H]D_3$ bound/DNA-cellulose pellet†	Specific pg $1,25(OH)_2[^3H]D_3$ bound/0.15 mg cytoplasmic protein‡
None	8.94 ± 0.25 (100%)	9.40 ± 0.43 (100%)
ATA, 2.5×10^{-5} M	0.22 ± 0.11§ (2.4%)	9.14 ± 0.27 (97.2%)
None + dialysis	7.80 ± 0.89 (100%)	8.63 ± 4.56 (100%)
ATA, 2.5×10^{-5} M, + dialysis	6.82 ± 0.71 (87.5%)	8.73 ± 0.59 (101.1%)
None	8.88 ± 1.10 (100%)	9.39 ± 0.58 (100%)
Rifamycin AF/013, 2×10^{-4} M	0.14 ± 0.09§ (1.6%)	8.68 ± 4.81 (92.4%)
None + dialysis	8.07 ± 0.51 (100%)	8.64 ± 0.26 (100%)
Rifamycin AF/013, 2×10^{-4} M, + dialysis	8.82 ± 0.43 (109.3%)	9.37 ± 0.87 (108.4%)

* Chicken intestinal cytosol (6.4 mg/ml) was incubated with 2.0 nM $1,25(OH)_2$[26,27-^{3}H]D_3 (158 Ci/mmole) as described in Materials and Methods. The labeled cytosol was divided; portions were untreated or incubated with ATA or rifamycin AF/013. Afterwards, aliquots of each treatment group were dialyzed against 500 vol. of TMDK-0.3 at 0–4° for 3.0 hr.

† DNA binding was assessed by incubating aliquots of $1,25(OH)_2[^3H]D_3$-labeled cytosol (0.15 mg protein) with DNA-cellulose as described in Materials and Methods. Values represent the means ± S.D. of triplicate determinations. Values in parentheses represent percent of control data.

‡ $1,25(OH)_2[^3H]D_3$ binding was quantitated by incubating aliquots of labeled cytosol (0.15 mg protein) with HAP as described in Materials and Methods. Values represent the means ± S.D. of triplicate determinations. Values in parentheses represent percent of control data.

§ Values are significantly different compared to cytosol not treated with inhibitors, $P < 0.05$.

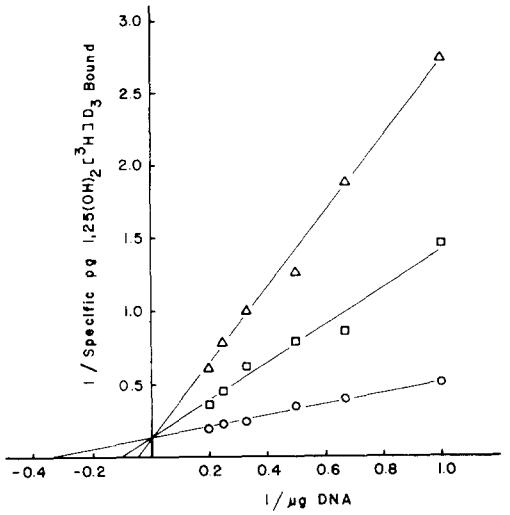

Fig. 2. Kinetics of inhibition of formation of $1,25(OH)_2$ [^{3}H]D_3–receptor–DNA-cellulose complexes by ATA and rifamycin AF/013. Chick intestinal cytosol (6.4 mg protein/ml) was labeled with 2.0 nM $1,25(OH)_2$[26,27-^{3}H]D_3 (158 Ci/mmole) as described in Materials and Methods. After the incubation period, the unbound sterol was removed by adsorption to dextran-coated charcoal, and the labeled cytosol was diluted to 0.03 M KCl. For the binding assay, aliquots of DNA-cellulose suspension (1–5 µg of calf thymus DNA/pellet) (total binding) or cellulose (nonspecific binding) were prepared as described in Materials and Methods. To the packed DNA-cellulose pellets were added the reaction mixtures (0.5 ml) in the presence of ATA (2×10^{-6} M) (△), rifamycin AF/013 (1.75×10^{-5} M) (□), or buffer alone (○). Values represent the mean for duplicate determinations.

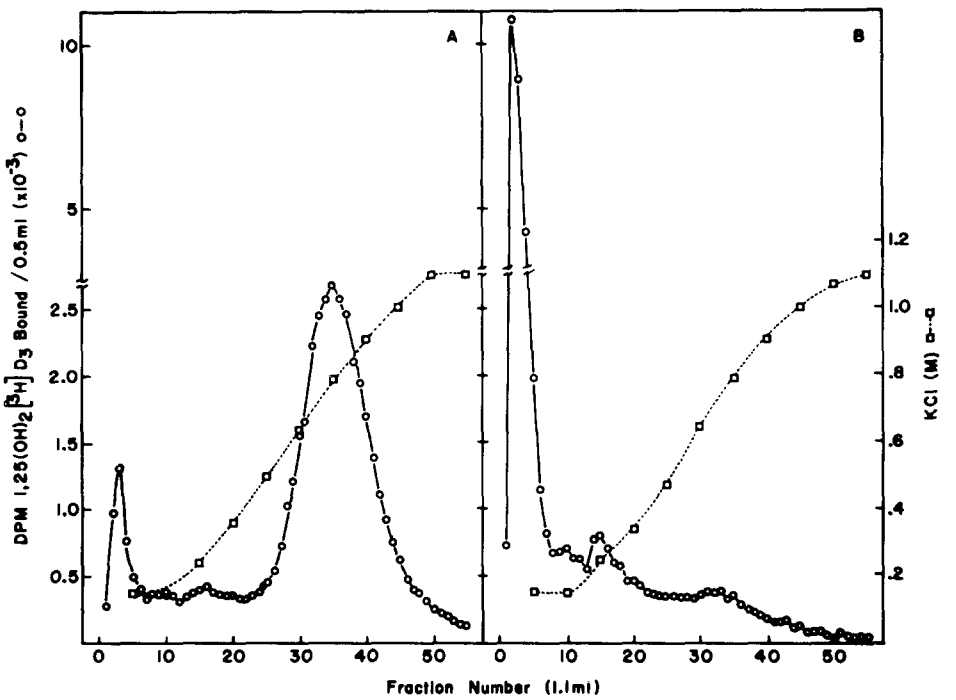

Fig. 3. Effect of ATA on the chromatographic behavior of $1,25(OH)_2$[^{3}H]D_3–receptor complexes utilizing Cibacron blue-agarose. Chicken intestinal cytosol (4.9 mg protein/ml) was incubated with 2.0 nM $1,25(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmole) as described in Materials and Methods. The labeled cytosol was divided; one portion was incubated in the presence of ATA (1×10^{-4} M) (panel B), and the remaining portion was incubated with an equal portion of TMD (panel A). After dextran-coated charcoal treatment, aliquots were diluted to a final KCl concentration of 0.15 M and applied to a Cibacron blue-agarose column (0.9 × 3.1 cm). The column was eluted with several volumes of TMDK-0.15 followed by a linear KCl gradient (0.15 to 1.50 M) at a flow rate of 0.8 ml/min. Fractions (1.1 ml) were collected, and 0.5 ml from each fraction was assayed for radioactivity. The salt gradient was measured by conductivity. Recovery of radioactivity for control and ATA-treated cytosol was 79 and 81% respectively.

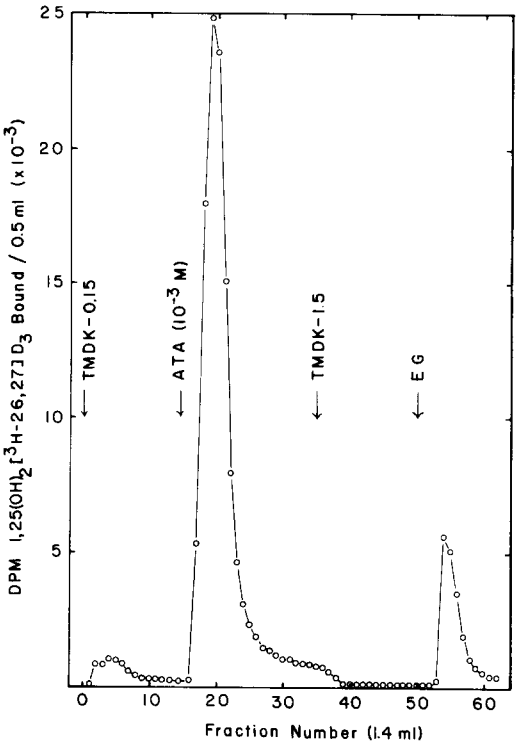

Fig. 4. Elution of 1,25(OH)$_2$[^{3}H]D$_3$–receptor complexes from Cibacron blue-agarose by ATA. Chicken intestinal cytosol (4.2 mg protein/ml) was labeled with 2.0 nM 1,25(OH)$_2$[26,27-^{3}H]D$_3$ (158 Ci/mmole) as described in Materials and Methods. After dextran-coated charcoal treatment, the labeled cytosol was diluted to a final KCl concentration of 0.15 M and applied to a Cibacron blue-agarose column (1.1 × 3.7 cm). The column was eluted at a flow rate of 0.8 ml/min with successive additions of TMDK-0.15, TMDK-0.15 containing ATA (10^{-3} M), TMDK-1.50, and finally stripped with ethylene glycol–TMDK-0.15 (3:2, v/v) (EG). Fractions (1.4 ml) were collected, and 0.5 ml from each tube was assayed for radioactivity. Recovery of radioactivity from the column was >98%.

ligand matrix. Cibacron blue-agarose binds labeled cytosol avidly. Successive elutions with TMDK-0.15, containing ATA (10^{-3} M), TMDK-1.5, and ethylene glycol-TMDK-0.15 (3:2, v/v) resulted in greater than 95% of the specifically bound 1,25(OH)$_2$D$_3$ eluting with the addition of ATA (Fig. 4). The radioactivity eluting with the ethylene glycol was not displaceable by a 200-fold excess of nonradioactive 1,25(OH)$_2$D$_3$ (data not shown). This has been noted previously for several immobilized dye-ligands [25].

The synthetic polyanionic resin, phosphocellulose, was utilized to describe further the interaction of ATA and rifamycin AF/013 with 1,25(OH)$_2$D$_3$–receptor complexes formed at 0–4°. Cytosol labeled with 1,25(OH)$_2$[^{3}H]D$_3$ was applied to a phosphocellulose column and chromatography was initiated with TMDK-0.03 followed by a linear KCl gradient (0.03 to 0.60 M). Receptor complexes were eluted maximally with a KCl concentration of 0.2 M (Fig. 5). Incubation of rifamycin AF/013 with 1,25(OH)$_2$D$_3$–receptor complexes altered the chromatographic profile dramatically (Fig. 5). Greater than 96% of the specifically labeled cytosol eluted in the drop-through fractions. A similar result was observed when ATA (5×10^{-5} M) was incubated with 1,25(OH)$_2$[^{3}H]D$_3$–receptor complexes followed by chromatography on phosphocellulose.

The amphoteric behavior of 1,25(OH)$_2$D$_3$–receptor complexes was demonstrated by chromatography on DEAE-cellulose, a cationic resin. Labeled cytosol applied to a DEAE-cellulose column resulted in retention of receptor which was eluted maximally at 0.14 M KCl (data not shown). However, neither ATA (5×10^{-5} M) nor rifamycin AF/013 (2×10^{-4} M) treatment was capable of significantly altering the elution profile (data not shown). This lack of effect subsequently was discovered to be due to retention of these inhibitors by DEAE-cellulose, thereby preventing an accurate assessment of the ATA and rifamycin AF/013 receptor interaction.

The integrity of 1,25(OH)$_2$D$_3$–receptor complexes in the absence or presence of ATA or rifamycin AF/013 was examined by sucrose density gradient analysis. Chicken intestinal cytosol was labeled with 1,25(OH)$_2$[^{3}H]D$_3$ and additionally incubated in the absence or presence of ATA (2.5×10^{-5} M) or rifamycin AF/013 (2×10^{-4} M). Aliquots of cytosol were applied to linear (4–20%) sucrose gradients containing the appropriate concentration of inhibitor. Control cytosol sedimented with a characteristic coefficient of 3.7 S (fraction no. 21) (Fig. 6A). A parallel incubation containing a 200-fold excess of nonradioactive sterol revealed that the 3.7 S macromolecule was labeled specifically. Incubation with ATA or rifamycin AF/013 shifted the migration of the receptor complex slightly (fraction no. 22) (panels B and C of Fig. 6). In addition, it is apparent that in the presence of rifamycin AF/013 there is a smaller peak of specifically bound 1,25(OH)$_2$[^{3}H]D$_3$ that sediments in the 6.0–7.0 S region as well as some accumulation in the pellet at the bottom of the tube. Also notable is the breadth of the gradient profile of the rifamycin AF/013-treated cytosol and the appearance of increased nonspecific binding (Fig. 6C). Increased nonspecific binding also occurs in the 3.7 S and 6.0 S regions of gradient profiles of chick liver cytosol that was labeled with 1,25(OH)$_2$[^{3}H]D$_3$ at 0–4° and subsequently incubated with rifamycin AF/013 (2×10^{-4} M) (data not shown). Although it appears that with rifamycin AF/013 treatment (Fig. 6C) there was loss of bound sterol when compared to the control profile (Fig. 6A), the aliquots of radioactivity added and recovery from the gradients were almost identical. These results were consistent for each of three separate sucrose density gradient experiments utilizing rifamycin AF/013.

When nuclear 1,25(OH)$_2$[^{3}H]D$_3$–receptor complexes were incubated with ATA (5×10^{-6} M) or rifamycin AF/013 (5×10^{-5} M), the sucrose density gradient profiles attained were similar to those using cytoplasmic receptor (data not shown). Most especially, nuclear receptor treated with rifamycin AF/013 displayed a broadened 3.7 S sedimenting macromolecule(s) with a discernible increase in nonspecific binding and a large accumulation of radioactivity in the pellet.

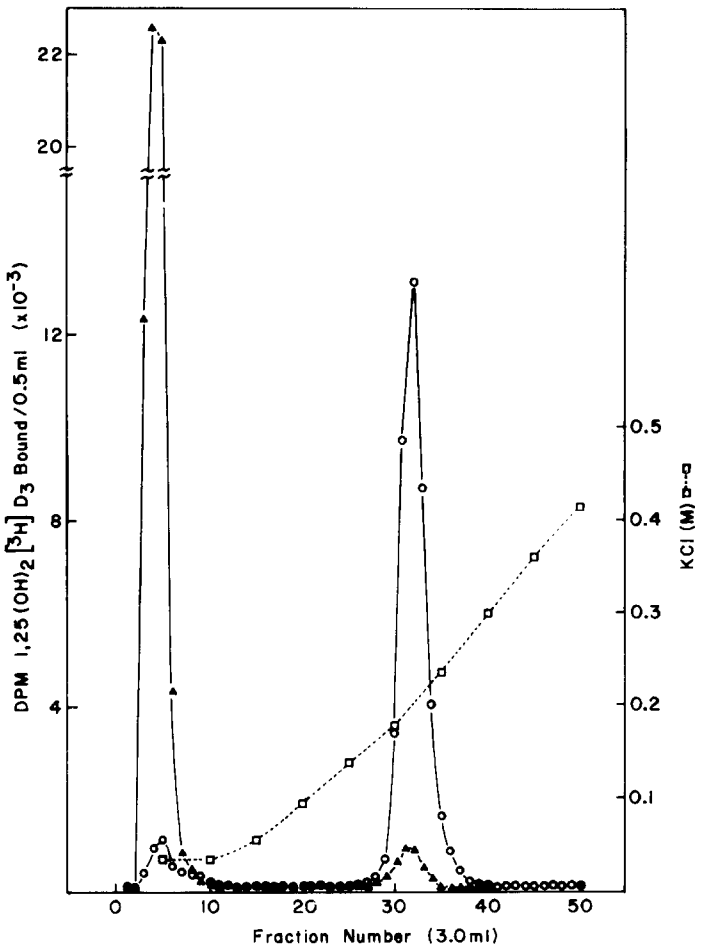

Fig. 5. Phosphocellulose chromatography of untreated and rifamycin AF/013-treated $1,25(OH)_2[^3H]D_3$–receptor complexes. Chicken intestinal cytosol (6.4 mg protein/ml) was incubated with 2.0 nM $1,25(OH)_2[26,27\text{-}^3H]D_3$ (158 Ci/mmole) as described in Materials and Methods. The labeled cytosol was either untreated (TMD buffer alone) (○) or incubated in the presence of rifamycin (AF/013 (2.2×10^{-4} M) (▲). After dextran-coated charcoal treatment, aliquots were diluted to a final KCl concentration of 0.03 M and applied to a phosphocellulose column (1.8 × 3.5 cm). The column was eluted with several volumes of TMDK-0.03 followed by a linear KCl gradient (0.03 to 0.60 M) at a flow rate of 0.9 ml/min. Fractions (3.0 ml) were collected, and 0.5 ml from each fraction was assayed for radioactivity. The salt gradient was measured by conductivity. Recovery of radioactivity for control and rifamycin AF/013-treated cytosol was 93 and 109% respectively.

DISCUSSION

Evidence has been presented that nuclear and DNA-cellulose binding to $1,25(OH)_2D_3$–receptor complex can be inhibited in a dose-dependent fashion by ATA and rifamycin AF/013. These results agree with published effects of ATA on the nuclear uptake of progesterone [17, 26] and estradiol receptors [16] and the inhibitory action of rifamycin AF/013 on the nuclear binding of progesterone receptors [15] and DNA-cellulose binding of activated glucocorticoid receptors [18]. It is not clear at this time as to the precise mechanism whereby these inhibitors prevent $1,25(OH)_2D_3$–receptor complex and other steroid hormone receptor binding to nuclear components and DNA-cellulose. ATA, a triphenylmethane dye, has been shown to inhibit both initiation and elongation processes of protein synthesis in cell-free extracts utilizing viral mRNA [27–29]. In addition, Grollman and Stewart [27] have postulated that ATA specifically functions by inhibiting mRNA to ribosomes. ATA and structurally related compounds also reduce specifically the affinity of the RNA-directed DNA polymerase for the DNA primer molecule [30] and interfere with the DNA-directed RNA polymerases [31]. The minimal structural requirements for this inhibitory activity in triphenylmethane compounds (i.e. ATA) are free or esterified catechol functions in two of the three aromatic nuclei and a sulfonic or carboxylic acid group in the third aromatic ring [31]. Similar requirements also have been reported for ATA and structurally related compounds that inhibit progesterone binding to isolated nuclei [26].

Rifamycin AF/013, a semisynthetic lipophilic derivative of rifamycin SV, has been reported to interfere in the binding of both RNA and DNA polymerase activities [32–35]. Whereas a related

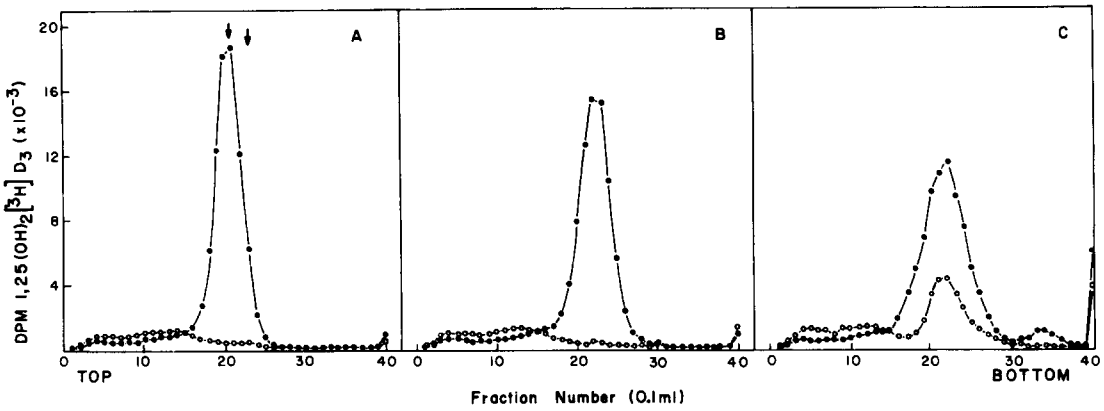

Fig. 6. Sedimentation behavior of ATA- and rifamycin AF/013-treated $1,25(OH)_2[^3H]D_3$–receptor complexes on sucrose density gradients. Chicken intestinal cytosol (6.4 mg protein/ml) was labeled with 2.0 nM $1,25(OH)_2[26,27\text{-}^3H]D_3$ (158 Ci/mmole) in the presence (○) or absence (●) of a 200-fold excess of nonradioactive $1,25(OH)_2D_3$ at 0–4° for 5.0 hr. Subsequently, the labeled cytosol was incubated with TMDK-0.3 (control) (A), ATA (2.5×10^{-5} M) (B), or rifamycin AF/013 (2×10^{-4} M) (C). Unbound sterol was removed by dextran-coated charcoal treatment. Aliquots of the supernatant fractions were applied to sucrose gradients (4–20%) containing TMDK-0.15 in the presence or absence of the appropriate concentration of inhibitor. Arrows indicate the position of marker proteins; ovalbumin, 3.7 S; bovine serum albumin, 4.4 S. Recovery of radioactivity for all gradients was >92%.

antibiotic, rifampicin, binds specifically and at very low concentrations to bacterial DNA-directed RNA polymerase [36], it has minimal, if any, effect on eukaryotic polymerases [32, 35]. Moreover, rifampicin does not prevent progesterone receptor binding to nuclei [15]. In this respect, the lack of inhibition by rifampicin on the binding of $1,25(OH)_2D_3$–receptor complexes to DNA-cellulose (Fig. 1) is in agreement with the previous findings involving the progesterone receptor. The observed difference between rifampicin and rifamycin AF/013 inhibition of receptor-DNA-cellulose binding may be attributed to the flexible lipophilic side-chain of rifamycin AF/013. This might make the binding of rifamycin AF/013 to a hydrophobic region of the receptor molecule necessary for DNA binding. Hydrophobic interaction of $1,25(OH)_2D_3$–receptor complex binding to double-stranded DNA has been suggested previously [11] as a primary mode of binding.

The concentration of rifamycin AF/013 necessary to inhibit 50% of the cytoplasmic-associated $1,25(OH)_2D_3$–receptor complex (1.2×10^{-4} M) from binding to DNA-cellulose is on the same order of potency for other steroid hormone receptors [15, 18]. In contrast, the dose necessary for inhibition of nuclear-associated $1,25(OH)_2D_3$–receptor complexes is several-fold lower (1.4×10^{-5} M) and is within the same order of magnitude for inhibition of polymerase activity [32–34]. There are several possibilities that could account for the observed increase in potency for rifamycin AF/013 induced inhibition of nuclear-associated receptor binding to DNA-cellulose. It was apparent after sucrose density gradient centrifugation for both nuclear- and cytoplasmic-associated receptors that rifamycin AF/013, an orange-colored compound, banded in regions where receptor was absent. Thus, if rifamycin AF/013 binds to other proteins, this may alter effectively its potency for inhibiting $1,25(OH)_2D_3$ receptors. Since our crude cytoplasmic preparations generally had a four to eight times higher protein concentration than nuclear extracts, rifamycin AF/013 extra-receptor binding might have been greater in the cytosol and effectively reduced the inhibitor concentration capable of interacting with $1,25(OH)_2D_3$ receptors. Second, the greater susceptibility of nuclear receptors to rifamycin AF/013 inhibition could have been due to inherent properties of these receptors. Although there does not appear to be an "activation" step involved in $1,25(OH)_2D_3$ receptor binding to DNA *in vitro* [11], this has not been entirely eliminated as a possibility. Previously, it has been reported [9] that $1,25(OH)_2D_3$ aporeceptors reside only in the nucleus when isolated under low ionic strength conditions. This observation is in marked contrast to results obtained with other steroid hormone aporeceptors which remain in the cytosol [37]. Thus, further studies using specific polymerase inhibitors to probe cytoplasmic- and nuclear-associated $1,25(OH)_2D_3$ receptors may provide information regarding possible differences in these forms.

From the results of this present investigation, it is apparent that ATA and rifamycin AF/013 interact with the receptor molecule and not DNA. This is consistent with results with polymerase inhibition studies and other steroid hormone receptors [15, 17, 18, 27, 31, 32]. $1,25(OH)_2D_3$–receptor complex binding to DNA-cellulose was not affected adversely by ATA or rifamycin AF/013 if the inhibitor was incubated with DNA-cellulose, but washed several times prior to receptor addition (Table 1). Therefore, the inhibitors must be present with receptor to prevent DNA binding. The inhibition by these compounds was largely reversible. Dialysis of receptor–inhibitor complexes resulted in 88% and >100% recovery of the DNA-cellulose binding for ATA- and rifamycin AF/013-treated cytosol respectively (Table 3). These results indicate that the receptor–inhibitor complex is fully dissociable, or that inhibi-

tion can occur only with the receptor–DNA complex. However, the kinetic experiment would suggest that both ATA and rifamycin AF/013 inhibition is competitive with respect to DNA binding. The site of inhibition could be directly at the polynucleotide domain or via an allosteric mechanism. Nevertheless, the inhibition of receptor binding to DNA-cellulose by ATA and rifamycin AF/013 is mutally exclusive. A replot of the slope of several reciprocal plots versus the corresponding inhibitor concentration was a straight line (results not shown). This favors the hypothesis that these polymerase inhibitors are highly competitive with respect to DNA at the polynucleotide domain of the receptor complex. It also argues that these inhibitors are interacting with the receptor and not the receptor–DNA complex. If ATA and rifamycin AF/013 inhibition of the receptor–DNA complex were occurring, an uncompetitive interaction might be expected.

Previously, Mellon [38] demonstrated that Cibacron blue interacts with the polynucleotide domain of the $1,25(OH)_2D_3$–receptor complex. Moreover, kinetic studies revealed that the interaction of Cibacron blue and DNA at the polynucleotide domain is mutally exclusive. Thus, the inhibition of Cibacron blue-agarose interaction with $1,25(OH)_2D_3$–receptor complexes by ATA and rifamycin AF/013 further supports the contention that these polymerase inhibitors can affect the polynucleotide domain (Fig. 3). Aside from blocking receptor complex binding to Cibacron blue-agarose, ATA also could elute receptor complexes previously bound to the resin (Fig. 4). Thus, ATA may be interacting within a site on the receptor that binds Cibacron blue and/or at a site that is common to both Cibacron blue and ATA, but not totally inclusive to both. Furthermore, the data could be interpreted such that ATA may be binding at a site allosterically altering the dye-ligand binding site. Similar findings have been reported for elution by ATA of progesterone receptors from an ATP-Sepharose column [26].

The present studies show further that these inhibitors affect selectively ionic groups on the receptor. Both ATA and rifamycin AF/013 completely disrupted receptor binding to the anion exchange resin phosphocellulose (Fig. 5). It has been postulated previously that the interaction of $1,25(OH)_2D_3$–receptor complexes with double-stranded DNA involves electrostatic and hydrophobic binding [11]. The electrostatic interaction could be explained by a region of positive charges on the receptor surface reacting with the phosphate groups associated with nucleotides. Therefore, the inhibitory action of ATA and rifamycin AF/013 on phosphocellulose and DNA binding suggests that these compounds may alter the positive charges on the receptor and/or induce a conformational change that restricts receptor access to anionic groups such as phosphate.

The stability of the $1,25(OH)_2D_3$–receptor complex to ATA and rifamycin AF/013 was examined by sucrose gradient centrifugation (Fig. 6). Rifamycin AF/013 treatment of cytoplasmic receptors resulted in the appearance of a small peak of specifically bound $1,25(OH)_2[^3H]D_3$ in the 6.0 S region of the gradient and an accumulation of radioactivity in the pellet which was consistent in three separate experiments. Moreover, nuclear-associated receptor treated in a similar manner also showed a significant accumulation of specifically bound $1,25(OH)_2[^3H]D_3$ in the pellet. The appearance of these faster migrating macromolecules may have been a result of receptor aggregation, although $1,25(OH)_2D_3$ receptors usually do not aggregate at 0.15 M KCl [39]. Previously, ATA has been shown to increase the sedimentation of single ribosomes [27]. Our present results do not indicate any strong aggregate effect of ATA on $1,25(OH)_2D_3$ receptors. It is unlikely that these faster sedimenting macromolecules caused the loss of DNA binding. At the concentration of rifamycin AF/013 used (2×10^{-4} M), there was no loss of sterol binding as measured by HAP assay, while DNA-cellulose binding was inhibited by 70–80% (Fig. 1). In addition, inhibition of DNA-cellulose binding occurred after a 1-hr rifamycin AF/013 incubation, whereas the receptor in the gradients was in contact with rifamycin AF/013 for 18–20 hr. Thus, the extent of the observed inhibition of DNA-cellulose binding cannot be accounted for by destruction of the $1,25(OH)_2D_3$–receptor complex.

The appearance of increased nonspecific binding upon rifamycin AF/013 treatment of cytoplasmic receptors (Fig. 6C) was also apparent with nuclear-associated receptors (data not shown). Moreover, $1,25(OH)_2[^3H]D_3$ labeling of chick liver cytosol, which does not contain $1,25(OH)_2D_3$ receptors, displayed the characteristic 6.0 S sedimenting macromolecule [40, 41]. Upon rifamycin AF/013 incubation, the nonspecific binding was increased significantly in the 6.0 S region and, also, had a small accumulation in the 3.7 S region. Thus, rifamycin AF/013 interacted with non-receptor proteins in a manner which resulted in nonspecific $1,25(OH)_2D_3$ binding. It should be emphasized that increased nonspecific binding was never observed in the HAP and DNA-cellulose procedures. Thus, this phenomenon may involve a time-dependent process with some ubiquitous macromolecule in cytosol and nuclear extracts.

The data presented here indicate that the observed inhibition of $1,25(OH)_2D_3$–receptor complex binding to DNA-cellulose by ATA and rifamycin AF/013 directly involved interaction of inhibitor with receptor. It also suggests that the receptor and polymerases had corresponding properties. This theory also has been advanced for several steroid hormone receptors [16, 19]. However, the question still remains, is this an insubstantial similarity or is it structurally relevant to hormone action?

Acknowledgements—The author thanks David Hinke and Rosanne Kosson for their expert technical assistance and Joni Dick for preparation of the manuscript.

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