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Modulators of the Glucocorticoid Receptor Also Regulate Mineralocorticoid Receptor Function†

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ABSTRACT: Modulators are proposed to be novel ether aminophosphoglycerides that stabilize unoccupied and occupied glucocorticoid receptor steroid binding and inhibit glucocorticoid receptor complex activation. Two isoforms, modulator 1 and modulator 2, have been purified from rat liver cytosol [Bodine, P. V., & Litwack, G. (1990) *J. Biol. Chem. 265*, 9544–9554]. Since the mineralocorticoid receptor is relatively resistant to activation, modulator's effect on rat distal colon mineralocorticoid receptor function was examined. Warming of unoccupied receptor decreased residual specific [^{3}H]aldosterone binding by 86 ± 2%. Both modulator isoforms completely prevented this destabilization with K_m's of 2 ± 1 μM modulator 1 and 24 ± 5 μM modulator 2. Warming of occupied mineralocorticoid receptors decreased [^{3}H]aldosterone binding by 56 ± 3%. Modulator only partially stabilized occupied receptor binding with K_m's of 10 ± 2 μM modulator 1 and 68 ± 8 μM modulator 2. Modulator inhibited receptor activation with K_m's of 3 ± 1 μM modulator 1 and 33 ± 10 μM modulator 2. Double-reciprocal analysis showed linear kinetics, and mixing modulator isoforms together had additive effects on unoccupied and occupied receptor steroid binding stabilization and activation inhibition. Colon cytosol contained a low molecular weight, heat-stable factor(s) which inhibited receptor activation and stabilized occupied receptor steroid binding. Molybdate completely stabilized unoccupied mineralocorticoid receptor steroid binding and inhibited activation with half-maximal effects at 3–4 mM but only stabilized occupied receptor binding by ~40%. These data indicate that (i) apparent physiologic concentrations of modulator stabilize mineralocorticoid receptor steroid binding and inhibit receptor activation, (ii) an aldosterone-responsive tissue contains a modulator-like activity, and (iii) molybdate mimics the effects of modulator. Thus, modulator affects both mineralocorticoid and glucocorticoid receptor function. Differences observed suggest that in vivo both modulator 1 and 2 could stabilize unoccupied mineralocorticoid receptor binding whereas only modulator 1 stabilizes unoccupied glucocorticoid receptor binding and that the modulator isoforms have additive effects on occupied mineralocorticoid receptor binding and activation but act synergistically on occupied glucocorticoid receptor binding and activation.

The steroid hormone receptors are a family of hormone-dependent transcriptional regulatory factors (Gustaffson et al., 1987). The unactivated steroid-binding receptor form is an oligomer containing receptor protein and the 90-kDa heat shock protein (hsp-90) (Joab et al., 1984). Binding of hormone to receptor induces dissociation of hsp-90, exposing the receptor DNA-binding domain, a process termed activation or transformation (Munck et al., 1990). The activated receptor then binds to specific hormone regulatory elements on DNA and alters transcription of steroid-responsive genes. The receptor subsequently dissociates from chromatin in a form unable to bind hormone and may be either degraded or recycled back to the oligomeric unactivated steroid-binding receptor form (Bodine & Litwack, 1990a).

Studies from many laboratories have demonstrated that each step in the steroid receptor cycle is influenced by other cellular factors (Bodine & Litwack, 1990a). Several endogenous steroid receptor regulators have been identified by their effects on a specific steroid receptor (Schmidt & Litwack, 1982; Dahmer et al., 1984), but it is unknown whether these factors affect all steroid hormone receptors or are specific for individual receptors. In contrast, a low molecular weight, heat-stable factor which stabilizes glucocorticoid receptor (GR) hormone binding and inhibits GR activation was subsequently reported to be present in many tissues and also to regulate estrogen and androgen receptor function (Dahmer et al., 1984; Cake et al., 1976; Leach et al., 1982; Sato et al., 1980). This

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factor has been termed modulator (Bodine & Litwack, 1990a). Our laboratory has reported the purification of modulator from rat liver cytosol and proposed that it is a novel ether amino-phosphoglyceride (Bodine & Litwack, 1988a,b). Recently, we identified two isoforms of modulator and demonstrated that the modulators had additive effects on unoccupied GR steroid-binding stabilization but acted synergistically to stabilize the occupied GR and to inhibit GR activation (Bodine & Litwack, 1990b). Purified modulator also increased the ability of purified activated protein kinase C to phosphorylate histones (Hsu et al., 1991). Meshinchi et al. (1988) identified a low molecular weight, heat-stable regulator of GR function in rat liver and L cell cytosol which they proposed to be a molybdenum-containing anion that appeared to be distinct from modulator (Bodine & Litwack, 1990b). Consequently, since there may be more than one low molecular weight substance which influences GR steroid binding and activation and since earlier studies used unpurified preparations of these factors, previous reports that several steroid receptors responded to this low molecular weight, heat-stable factor may have actually reflected the presence of several different factors in cytosol (Dahmer et al., 1984; Leach et al., 1982; Sato et al., 1980).

Once purified modulator was obtained, it became possible to determine directly whether it also regulates steroid binding and activation of other steroid hormone receptors or if its effects were limited to GR function. In this study, we examined the effect of purified modulator on rat distal colon mineralocorticoid receptor (MR) properties. The MR was selected because it is relatively resistant to activation in vitro which could be explained by an interaction of modulator with the MR (Eisen & Harmon, 1986; Luttge & Emadian, 1988; Schulman et al., 1986). Moreover, the predicted amino acid sequences of the MR and GR are highly homologous, and therefore the MR and GR may respond similarly to modulator (Arriza et al., 1987).

Materials and Methods

Chemicals. D-[1,2,6,7-^{3}H]Aldosterone (82–92 Ci/mmol) was purchased from New England Nuclear. RU28362[1] was the gift of Roussel UCLAF, Paris, France. PD-10 (Sephadex G-25M) columns, Sephacryl S-300, and gel filtration protein standards were obtained from Pharmacia LKB Biotechnology Inc. DEAE-cellulose (Whatman DE52) was from Whatman. Phenylmethanesulfonyl fluoride (PMSF), sodium molybdate, and dithiothreitol were from Sigma. All other reagents were from Fisher.

Cytosol Preparation. Male Sprague-Dawley rats (100–200 g) were adrenalectomized under Nembutal anesthesia and maintained on rat chow and 0.9% saline. Two to five days after adrenalectomy, rats were anesthesized with 0.1 mL of Nembutal/100 g of body weight, and distal colon segments were removed as described previously (Schulman et al., 1986). Distal colon epithelial cells were scraped free with a glass slide and placed in 0.5 mL/intestinal segment of iced 50 mM Tris buffer, pH 7.6, containing 250 mM sucrose, 2 mM EDTA, 1 mM PMSF, 20 mM sodium molybdate, and 10 mM dithiothreitol (TSE buffer). Tissue was homogenized in a Teflon–glass Potter–Elvehjem apparatus for 8–10 strokes and centrifuged at 105000g for 60 min at 0–4 °C in a Beckman L5-50 ultracentrifuge. The supernatant cytosol was stored at −80 °C prior to use. The cytosolic protein concentration, determined by Bradford microassay, was typically 10–15 mg/mL (Bradford, 1976).

Binding of [^{3}H]Aldosterone. Total, specific, and nonspecific steroid binding were determined by incubation of cytosol with 50 nM [^{3}H]aldosterone, plus a 500-fold molar excess of the specific glucocorticoid RU28362 to prevent crossover binding of aldosterone to the glucocorticoid receptor, in the presence and absence of 500–1000-fold excess unlabeled aldosterone for 30 min at 25 °C. Samples (75 or 100 μL) were incubated with 400 μL of hydroxylapatite for 10 min at 0–4 °C and then washed three times with 2.5 mL of 50 mM potassium phosphate, pH 7.0, containing 10 mM sodium molybdate. The pellet was then transfered to scintillation vials, mixed with 5 mL of Scintiverse II (Fisher) and counted in a Beckman LS2800 liquid-scintillation spectrometer with a counting efficiency for tritium of approximately 60%. Previous studies indicated that specific [^{3}H]aldosterone equilibrium binding was stable at 25 °C for at least 90 min and greater than the specific binding obtained after incubation at 0–4 °C for 5 h (Schulman et al., 1986).

DNA-Cellulose Binding Assay. Binding of [^{3}H]aldosterone-bound receptor to calf-thymus DNA-cellulose was determined by the method of Kalimi et al. (1975) as previously described (Schulman et al., 1986). The percent binding to DNA-cellulose was calculated by dividing the dpm's of [^{3}H]aldosterone bound to DNA-cellulose by the dpm's bound to hydroxylapatite and multiplying by 100.

Purification of Modulator. Two isoforms of modulator were purified from rat liver cytosol as previously described (Bodine & Litwack, 1990b). In brief, cytosol was heated at 37 °C for 5 h in the presence of trypsin. The resulting supernatant was applied to a Sephadex G-15 gel filtration column. Two peaks of modulator activity were isolated. The peak eluting earliest, previously designated peak 1, will be referred to as modulator 1 (M1) and the peak eluting later, previously designated as peak 2, will be referred to as modulator 2 (M2). Both M1 and M2 were further purified by Dowex-1 anion exchange chromatography and estimated to be ≥95% pure by several methods. Purified modulator was dissolved in HPLC-grade H_2O, and aliquots were stored at −80 °C. Molar concentrations of each isoform were calculated by ninhydrin assay (amines), acid molybdate reactions (phosphate), and the dry weight of the purified sample, with estimated molecular weights as previously published (Bodine & Litwack, 1990b). The absolute structural determination and chemical synthesis of the modulators is currently underway.

Activation Inhibition and Stabilization of Occupied MR Assay. Aliquots of [^{3}H]aldosterone-labeled distal colon cytosol (1.5 mL) were filtered through 10-mL bed volume PD-10 columns (Sephadex G-25M) equilibrated with TSE buffer without molybdate. This step removes molybdate, free [^{3}H]aldosterone, and endogenous modulator from cytosol. Purified modulators 1 and 2 were added to 400 μL of filtered cytosol, and HPLC-grade H_2O was added to achieve a final volume of 500 μL. Receptor was then activated by warming at 25 °C for 30 min, the reaction was stopped by addition of 20 mM sodium molybdate, and duplicate 100-μL aliquots were removed for hydroxylapatite and DNA-cellulose binding assays. Two simultaneous controls were included. The molybdate (unactivated) control was cytosol subjected to gel filtration but not warmed, and 20 mM molybdate was added back immediately after gel filtration to prevent activation. The H_2O (activated) control was subjected to warming in the absence of modulator and molybdate. The percent binding to DNA-cellulose relative to control was calculated from the

[1] Abbreviations: RU28362, 11β,17β-dihydroxy-6-methyl-17-propionylandrosta-1,4,6-triene-3-one; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride.

following equation: 100 × [(sample − molybdate control)/(H_2O control − molybdate control)]. The percent occupied MR binding relative to control was calculated as detailed below.

Stabilization of Unoccupied MR Assay. Aliquots of unlabeled distal colon cytosol (1.5 mL) were chromatographed on PD-10 columns to remove endogenous modulator and molybdate, and the 3-mL void volume was pooled. Purified modulator was added to 400-μL aliquots of filtered cytosol as described above. Unoccupied cytosolic receptor was destabilized (inactivated) by incubation at 20 °C for 30 min. Following this, 20 mM molybdate was added to prevent further inactivation. Samples were then divided into two 250-μL aliquots. Residual total and nonspecific [^{3}H]aldosterone binding to receptor was measured in duplicate 100-μL aliquots by hydroxylapatite binding assay, and specific steroid binding was calculated. Simultaneous molybdate and water controls were included in each experiment. The specific [^{3}H]aldosterone binding relative to control was calculated from the following equation: 100 × [(sample − H_2O control)/(molybdate control − H_2O control)].

Column Chromatography. All chromatography was done at 4 °C. DEAE-cellulose anion exchange chromatography was performed as described previously using a 40-mL 0–500 mM KCl gradient, except that 10% monothioglycerol, 10 mM molybdate, and 1 mM PMSF were included in all buffers (Schulman et al., 1986). Ion concentration was determined by measurement of conductivity (Markson model 10 conductivity meter) using KCl standards of known ion concentration.

For size exclusion chromatography, 1-mL samples were applied to a 70 × 2.6 cm column of Sephacryl S-300 equilibrated in 50 mM potassium phosphate, pH 7.0, containing 400 mM KCl, 20 mM sodium molybdate, 10% monothioglycerol, and 1 mM PMSF. One-milliliter fractions were collected and analyzed for radioactivity using a Beckman LS2800 liquid-scintillation spectrometer. The void volume was determined by the elution of blue dextran and the total volume by the elution of free radiolabeled steroid. The apparent Stokes radius (R_s) of the receptor was determined from a standard curve of ($-\log K_{av}^{1/2}$) vs R_s of the protein standards thyroglobulin (85 Å), ferritin (61 Å), albumin (35.5 Å), chymotrypsin (20.9 Å), and ribonuclease A (16.4 Å).

Data Analysis. Results of steroid binding stabilization and activation inhibition assays are presented as the mean ± SEM. Double-reciprocal analysis was done to determine the kinetics of the interaction between modulator and the MR. If first-order (linear) curve fitting had a correlation coefficient $R \geq 0.95$, the linear model was assumed to describe the data. Apparent K_m's were calculated by directly fitting the dose–response data to a hyperbola using the following equation: $V = (V_{max} \times [\text{modulator}])/([\text{modulator}] + K)$ with V_{max} constrained to 100%. This approach was utilized to avoid inaccuracies inherent in estimating K_m by double-reciprocal analysis. These calculations were performed by Dr. Ronald Tallarida and Keith Freeman (Department of Pharmacology, Temple University School of Medicine). It is recognized that the K_m is only valid if modulator directly interacts with receptor and that this hypothesis has not yet been proved. However, calculation of K_m was performed to quantitatively compare results in this study with those previously obtained with the GR (Bodine & Litwack, 1990b). The concentration of modulator that resulted in 50% stabilization of steroid binding or 50% inhibition of activation was estimated from the dose–response curves (EC_{50}). As defined, if the maximal effect of modulator was less than 100%, the EC_{50} would not be the same as the concentration of modulator causing 50% of the observed effect. The EC_{50} was calculated this way to facilitate comparison of the effects of modulator on the MR vs the GR.

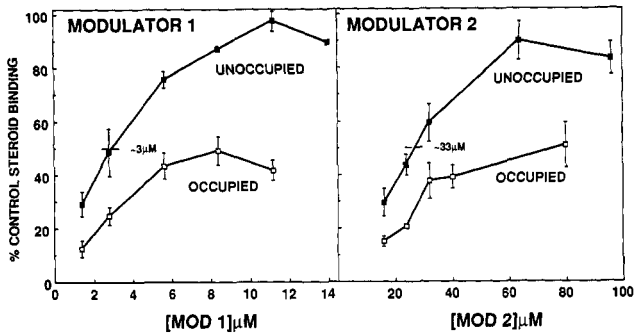

FIGURE 1: Dose–response curves for the effect of modulator on MR steroid binding stability. Purified modulator 1 (left panel) and modulator 2 (right panel) were tested for their ability to stabilize unoccupied MR (closed squares) and occupied MR (open squares) steroid binding as described under Materials and Methods. The occupied MR steroid binding stabilization data are derived from the hydroxylapatite portion of the activation inhibition assay. Each point represents the mean ± SEM of three to six measurements.

Results

Effect of Modulator on MR Steroid Binding Stability. Incubation of unbound receptor at 20 °C for 30 min destabilized (inactivated) 86 ± 2% of MR binding (from 10046 ± 1031 dpm/100 μL specific [^{3}H]aldosterone binding with 17 ± 1% nonspecific binding in the 0 °C molybdate control to 1354 ± 247 dpm/100 μL with 64 ± 5% nonspecific binding in the 20 °C H_2O control, $n = 10$). Both isoforms of modulator prevented destabilization in a dose-dependent manner (Figure 1). However, modulator 1 was approximately 10 times more potent than modulator 2 for this activity. Maximal binding stabilization was 97 ± 4% with 11 μM modulator 1 and 89 ± 8% with 64 μM modulator 2. Higher concentrations of each isoform (14 μM M1 and 96 μM M2) did not have a greater effect. This plateau in the degree of MR steroid binding stabilization is different than the previously reported biphasic response of the GR to modulator 1 and 2 where low doses of modulator stabilized GR steroid binding, while higher doses destabilized GR binding (Bodine & Litwack, 1990b).

Warming of steroid-bound MR decreased the amount of steroid-bound receptor to a lesser extent than the warming of the unoccupied MR. In the 0 °C molybdate control [^{3}H]-aldosterone binding averaged 8024 ± 966 dpm/100 μL (n = 12), whereas it decreased by 56 ± 3% to 3324 ± 475 dpm/100 μL after warming in the absence of molybdate or modulator. Modulator also stabilized occupied MR steroid binding in a dose-dependent fashion (Figure 1). However, in contrast to the effects of modulator on the unoccupied MR and the occupied GR (Bodine & Litwack, 1990b), only partial steroid binding stabilization of the occupied MR was observed (44 ± 1% stabilization with 8 μM modulator 1 and 51 ± 9% stabilization with 80 μM modulator 2).

Double-reciprocal plots of the data in Figure 1 were linear for both unoccupied and occupied MR stability (data not shown). This is consistent with a one-step or one-site interaction between modulator and the MR. The concentrations of modulator that stabilized steroid binding by 50% (EC_{50}) were estimated from the dose–response curves and compared to the calculated apparent K_m. The EC_{50} for unoccupied MR steroid binding stabilization was 3 and 33 μM and the K_m was 2.2 ± 0.5 and 23.8 ± 5.2 μM, for modulator 1 and 2, re-

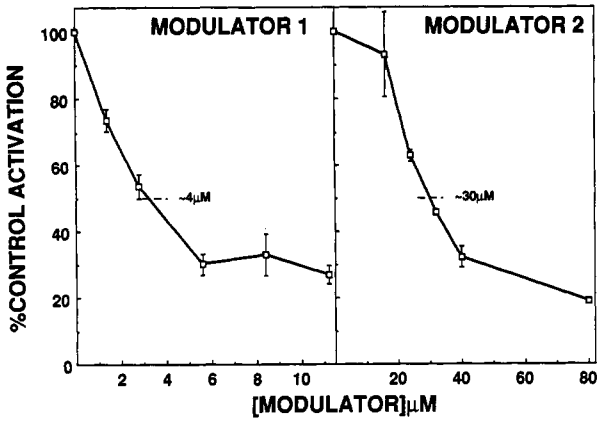

FIGURE 2: Dose–response curves for the effect of modulator on MR activation. Purified modulator 1 (left panel) and modulator 2 (right panel) were tested for their ability to inhibit MR activation as described under Materials and Methods. The data represent the mean ± SEM of three to six measurements.

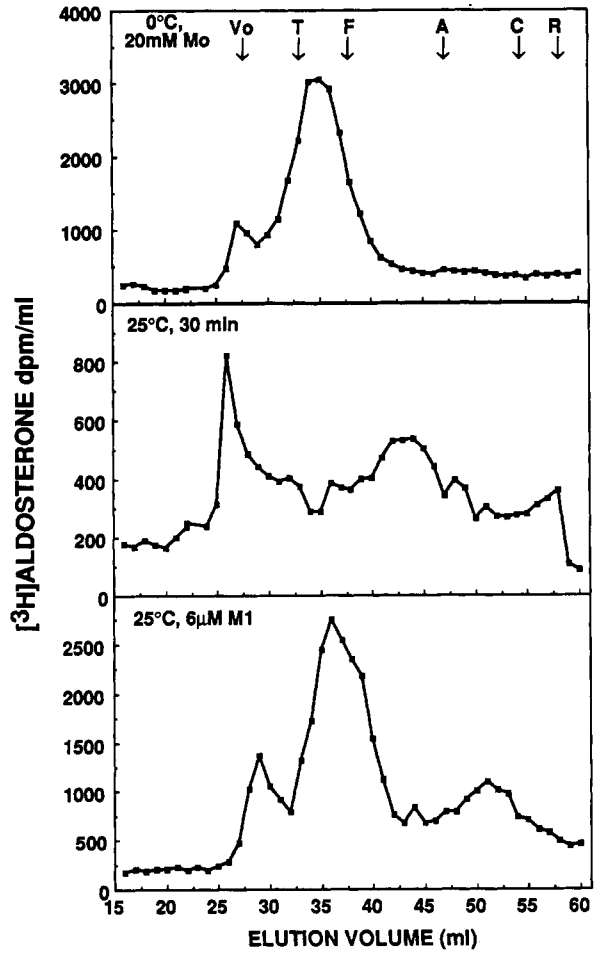

FIGURE 3: Sephacryl S-300 size-exclusion chromatography of the MR. [^{3}H]Aldosterone-labeled cytosol was chromatographed on Sephadex G-25M and incubated at 0 °C in the presence of 20 mM molybdate (top panel), at 25 °C for 30 min in the absence of molybdate or modulator (middle panel), or at 25 °C for 30 min in the presence of 6 μM modulator 1 (lower panel) prior to chromatography (see Materials and Methods for details). Unactivated MR (top and bottom panels) elutes at an R_s of 73 Å. Activated MR (middle panel) elutes at an R_s of 30–40 Å. The void volume (V_o) and elution volumes of the calibration standards thyroglobulin (T), ferritin (F), albumin (A), chymotrypsin (C), and ribonuclease A (R) are indicated by the arrows.

spectively. These are within the reported cellular ranges for each isoform in rat liver (Bodine & Litwack, 1990b). Two- to three-fold higher concentrations of modulator were required

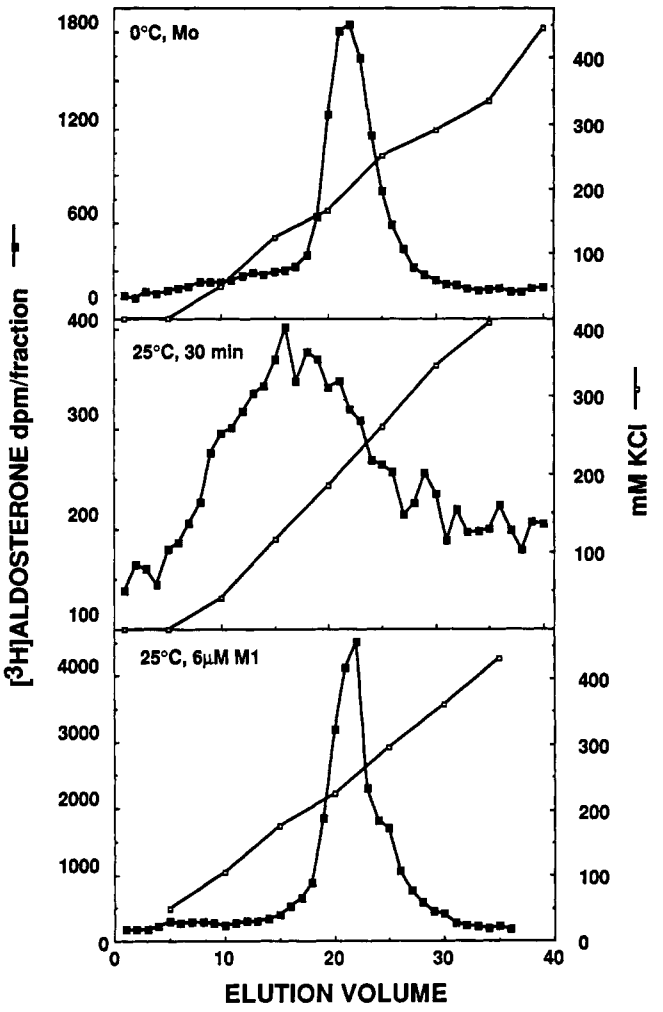

FIGURE 4: DEAE-cellulose anion exchange chromatography of the MR. [^{3}H]Aldosterone-labeled cytosol was filtered through a Sephadex G-25M column and incubated as described in the legend to Figure 3 prior to DEAE-cellulose chromatography.

to stabilize occupied MR steroid binding (EC_{50}, 9 and 64 μM; K_m, 9.9 ± 1.5 and 67.8 ± 8.0 μM, for modulators 1 and 2, respectively).

Effect of Modulator on MR Activation. Warming steroid-bound MR resulted in binding of 1627 ± 214 dpm/100 μL or 48 ± 4% of the MR to DNA-cellulose, compared to 901 ± 90 dpm/100 μL or 12 ± 2% DNA-cellulose binding for the unactivated control (n = 12). Modulators 1 and 2 both inhibited MR activation in a dose-dependent manner (Figure 2). Maximum activation inhibition was obtained with 6 μM modulator 1 (73 ± 2%, n = 3) and 80 μM modulator 2 (81 ± 1%, n = 4). Increasing the concentration of modulator 1 to 11 μM did not further inhibit activation. Examination of Figure 2 suggests that the effect of modulator 2 on MR activation plateaus between 40 and 80 μM. Double-reciprocal plots of the data in Figure 2 were linear (data not shown). Thus, as with steroid binding stabilization, the effect of modulators 1 and 2 on MR activation follows first-order kinetics. The EC_{50} was 4 and 30 μM, and the K_m was 3.4 ± 0.3 and 33.1 ± 10.1 μM, for modulators 1 and 2, respectively.

Size-exclusion chromatography (Figure 3) and DEAE-cellulose chromatography (Figure 4) were performed to ascertain whether modulator prevented the structural changes associated with activation. Chromatograms of unactivated MR (top panels) and of MR subjected to activation conditions in the absence (middle panels) and presence (lower panels) of maximally effective concentrations of modulator 1 (6 μM)

Table I: Effect of Gel Filtration on Occupied MR Steroid Binding and Activation[a]

conditions	unactivated binding (dpm/100 μL) to HAP	unactivated binding (dpm/100 μL) to DNA-c	unactivated % DNA-c	activated binding (dpm/100 μL) to HAP	activated binding (dpm/100 μL) to DNA-c	activated % DNA-c	% decrease in HAP binding
25 °C, 30 min (n = 4)	5951 ± 1394	475 ± 87	8 ± 1	4506 ± 1243	1149 ± 287	28 ± 6	23 ± 14
G25, 25 °C, 30 min (n = 12)	8024 ± 966	901 ± 90	12 ± 1	3324 ± 302	1627 ± 302	48 ± 2	56 ● 3

[a] Cytosol not containing molybdate was incubated with [^{3}H]aldosterone at 0–4 °C for 3 h and then MR activated by warming and compared to cytosol containing molybdate that was incubated with [^{3}H]aldosterone at 25 °C for 30 min and filtered through Sephadex G25 to remove molybdate and endogenous modulator prior to activation. Binding of [^{3}H]aldosterone to the MR was measured by hydroxylapatite adsorption (HAP), and MR binding to DNA was measured by DNA-cellulose (DNA-c) binding assay before and after activation. The percent decrease in HAP binding after activation is indicated in the last column.

are shown. As expected, unactivated MR had a Stokes radius (R_s) of 73 Å and eluted from DEAE-cellulose at 200 mM KCl, while activated MR was smaller (R_s 30–40 Å) and eluted from DEAE-cellulose with lower concentrations of KCl. Modulator prevented this shift in size and charge. Thus, modulator inhibited the structural changes commonly associated with activation. This confirms that modulator actually inhibits MR activation and that the decreased percent DNA-cellulose binding observed in the presence of modulator was not due simply to stabilization of occupied MR steroid binding or inhibition of activated MR binding to DNA.

Effect of Mixing Modulators 1 and 2. Submaximal concentrations of modulator 1 (1.5 and 3 μM) and modulator 2 (16 and 24 μM) were mixed together and the results compared with the effects of each alone. For each assay, the observed results were within 10% of those predicted if mixing the isoforms had additive effects.

Effect of Colon Cytosol on MR Function. Rat liver cytosol is the source of the modulator used in this study, but liver contains no MR (Schulman et al., 1986). Two experiments were performed to determine if mineralocorticoid target tissue contains modulator. First, distal colon cytosol was assayed for modulator activity. For these studies, molybdate-free cytosol was heated at 37 °C for 1 h to denature the MR, and the resultant supernatant was lyophilized and resuspended in TSE buffer. As a control, the supernatant was subjected to gel filtration on a Sephadex G–25 column prior to lyophilization in order to remove low molecular weight factors such as modulator. This receptor depleted cytosol (RDC) was then used in place of purified modulator in the MR activation inhibition assay. As shown in Figure 5, RDC contained a factor which stabilized occupied MR steroid binding and inhibited MR activation in a concentration-dependent manner. RDC also stabilized the occupied rat liver GR steroid binding and inhibited GR activation in a similar manner (data not shown). Most of this activity was removed by prior Sephadex G-25 gel filtration, demonstrating that it had a molecular mass <5000 kDa. Second, the effect of Sephadex G-25 gel filtration on MR activation and steroid binding was examined (Table I). Warming of MR resulted in higher absolute and percent DNA-cellulose binding in cytosol subjected to gel filtration before activation compared to non-gel-filtered cytosol. In addition, warming of MR decreased [^{3}H]aldosterone binding more after gel filtration. These data confirm that distal colon cytosol contains a low molecular weight, heat-stable factor with modulator-like activity.

Molybdate and MR Function. We have proposed that exogenous sodium molybdate mimics the effects of endogenous modulator on the GR with the exception that molybdate is ~100 times less potent than modulator (Bodine & Litwack, 1988b, 1990a,c). Although we and others had previously shown that molybdate inhibited MR activation, its effects on MR steroid binding stability had not been extensively examined (Schulman et al., 1986; Eisen & Harmon, 1986; Luttge & Emadian, 1988; Rafestin-Oblin et al., 1989). Therefore, molybdate was used in place of modulator in the MR steroid binding stabilization and activation inhibition assays. Figure 6 shows that molybdate completely stabilizes unoccupied MR steroid binding and inhibits MR activation in a dose-dependent manner. The concentration of molybdate which stabilized unoccupied MR steroid binding and inhibited MR activation by 50% was ~3–4 mM, indicating that molybdate is ~1000-fold less potent than modulator 1 and ~100-fold less potent than modulator 2. However, like modulator, molybdate was less effective at stabilizing occupied MR steroid binding vs unoccupied MR binding with only ~40% stabilization of the occupied MR even at 10–40 mM molybdate.

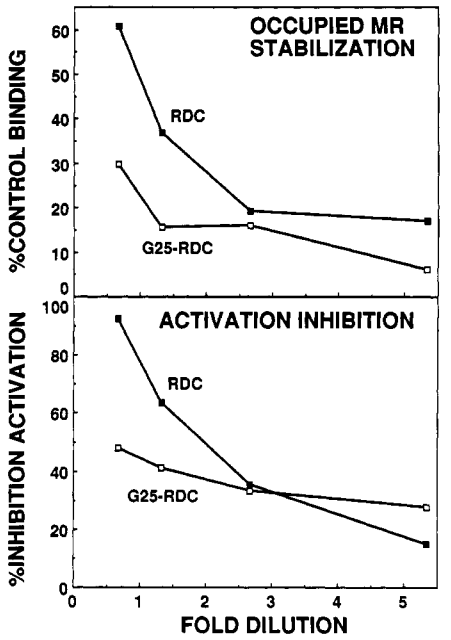

FIGURE 5: Effect of receptor-depleted cytosol (RDC) on occupied MR function. Distal colon cytosol was depleted of MR as detailed in the text and used before (closed squares) and after (open squares) filtration of RDC on Sephadex G-25M columns in place of purified modulator in the MR activation inhibition assay. Stabilization of occupied MR steroid binding is shown in the top panel and inhibition of MR activation is shown in the bottom panel. The fold dilution is relative to the original colon cytosol. The steroid binding stabilization data are derived from the hydroxylapatite portion of the activation inhibition assay (see Materials and Methods for details).

Discussion

These data indicate that modulator has profound effects on MR function. The similarities between the effects of modulator on the liver GR and the colon MR include stabilization of unoccupied and occupied receptor steroid binding and inhibition of receptor activation as assessed by DNA-cellulose binding, DEAE-cellulose chromatography, and size-exclusion chromatography (Bodine & Litwack, 1988a,b, 1990b). These similarities, and the evidence that modulator may also be

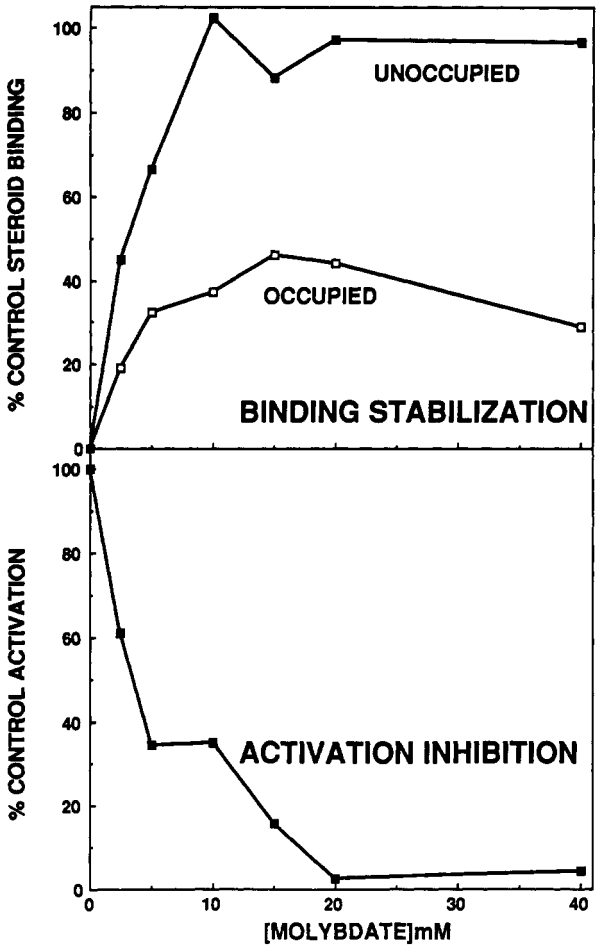

FIGURE 6: Dose–response curves for the effect of molybdate on MR stability and activation. Molybdate was used in place of modulator in the unoccupied MR steroid binding stabilization and activation inhibition assays. The top panel shows the effects of molybdate on stabilization of steroid binding for the unoccupied (closed squares) and occupied (open squares) MR. The bottom panel represents the effect of molybdate on MR activation. Results represent the mean of two experiments.

present in mineralocorticoid target cell cytosol, suggest that modulator could regulate the function of both receptors in vivo.

Modulator was discovered when it was noted that GR activation increased, and GR steroid binding stability decreased, after removal of low molecular weight molecules by gel filtration (Cake et al., 1976). We had reported that gel filtration did not promote MR activation (Schulman et al., 1986). However, in that study cytosol was homogenized in potassium phosphate (KP) rather than TSE buffer. Gel filtration does increase MR activation in cytosol homogenized in TSE (Table I). This implies either that removal of modulator does not promote MR activation in the absence of other conditions which favor activation (pH ≥7.5 and EDTA), or that modulator dissociates from its putative cytosolic binding protein in the presence of TSE but not KP. Since purified modulator inhibits MR activation, previous observations that the MR is resistant to activation may be due to endogenous modulator (Eisen & Harmon, 1986; Luttge & Emadian, 1988; Schulman et al., 1986).

Molybdate and modulator completely stabilize unoccupied MR steroid binding. The present study indicates that 50% stabilization of unoccupied MR steroid binding requires ~3 mM molybdate and maximal stabilization occurs with 10 mM molybdate. A previous study reported 50% effectiveness at ~0.2 mM and maximal effects at 0.5–1.0 mM molybdate (Emadian et al., 1988b). This discrepancy may reflect that in the present study endogenous modulator was removed by gel filtration, whereas the previous study utilized non-gel-filtered cytosol. The effects of modulator and molybdate are additive for GR function (Bodine & Litwack, 1988b), and endogenous modulator would therefore lower the apparent effective concentrations of molybdate.

Modulator and molybdate only stabilized occupied MR steriod binding by 40%. Emadian et al. (1986) found that 20 mM molybdate actually decreased occupied MR binding. Thus, it appears that other factors are required for maximum occupied MR stability. Our previous data indicated that, in non-filtered colon cytosol containing 20 mM molybdate, occupied MR was stable for 90 min at 25 °C (Schulman et al., 1986). However, since neither modulator nor molybdate completely stabilized occupied MR steroid binding in this study, additional determinants of MR steroid binding stability may be removed by gel filtration. It is unlikely that this factor is the molybdenum-containing anion isolated by Meshinchi et al. (1988, 1990) since molybdate is reported to mimic the effects of the endogenous molybdenum anion on the GR. This other factor may stabilize the activated MR. This hypothesis is supported by the observation that the activated MR was not stable during column chromatography (Figures 3 and 4), whereas in a previous study of MR activated in the absence of gel filtration, distinct activated MR peaks were observed (Schulman et al., 1986). The activated MR is known to be more labile than the activated GR (Eisen & Harmon, 1986; Emadian et al., 1986). If proteolysis of activated MR occurs and separates the steroid- and DNA-binding domains, then the DNA-binding fragments lacking steroid would not be detectable whereas the steroid-bound fragments would be measured by hydroxylapatite assay. This should result in underestimation of the percent activation and thus overestimate the ability of modulator and molybdate to inhibit MR activation. Limited proteolysis would not explain why modulator and molybdate do not more effectively stabilize occupied MR steroid binding. More extensive proteolysis of the activated MR resulting in dissociation of steroid would decrease steroid binding. This would account for the partial effectiveness of modulator and molybdate on occupied MR steroid binding stabilization and more complete inhibition of activation only to the extent that activation occurred. This possibility cannot be excluded until activated MR can be stabilized. Proteolysis of the occupied unactivated MR with loss of a modulator binding site might also result in a similar lack of stabilization by modulator and molybdate of occupied MR. This, however, would not explain the ability of these agents to inhibit activation at lower concentrations than those that inhibit GR activation. Unactivated MR proteolysis is also unlikely since MR warmed in the presence of modulator had the same R_s and elution pattern from DEAE-cellulose as unactivated MR (Figures 3 and 4).

Molybdate was slightly more effective than either modulator isoform in inhibiting activation of the MR. This discrepancy was not observed when GR was studied. It is probably not related to the inability of modulator to completely stabilize occupied MR steroid binding since molybdate and modulator did not differ in this regard. The mechanism of the difference in maximal inhibition of activation induced by molybdate and modulator requires further study.

A comparison of the effects of modulator on the colon MR and rat liver GR is shown in Table II. The GR data were previously published (Bodine & Litwack, 1990b). The same batch of purified modulator was used in both studies. The GR data have been modified since first published. Previous

Table II: Comparison of the Effect of Modulator 1 (M1) and Modulator 2 (M2) on MR vs GR[a]

	mineralocorticoid receptor				glucocorticoid receptor			
	M1	M2	kinetics	mixing	M1	M2	kinetics	mixing
unoccupied								
EC_{50} (μM)	3	33	linear	additive	9	51	linear	additive
K_m (μM)	2 ± 1	24 ± 5			9 ± 2	50 ± 4		
R	0.996	0.968			0.999	0.990		
occupied								
EC_{50} (μM)	9	64	linear	additive	13	73	polynomial	synergistic
K_m (μM)	10 ± 2	68 ± 8						
R	0.984	0.967			0.999	0.992		
activation								
EC_{50} (μM)	4	30	linear	additive	13	66	polynomial	synergistic
K_m (μM)	3 ± 0.3	33 ± 10						
R	0.989	0.951			1.000	0.998		

[a] The data for the rat liver GR are from a previous study (Bodine & Litwack, 1990b) except for the K_m's, which have been modified as discussed in the text. The concentrations of modulator causing 50% stabilization of steroid binding and 50% inhibition of activation (EC_{50}) were estimated from the dose–response curves. The K_m's were calculated as detailed under Materials and Methods for linear relationships. The correlation coefficients (R) were derived from double-reciprocal analysis.

analysis of unoccupied GR steroid binding stabilization revealed a discrepancy between the EC_{50} of 9 μM and the calculated K_m of 101 μM for modulator 1. Because the K_m was ~10-fold higher than the estimated physiologic concentration of modulator 1, we concluded that modulator 1 had no effect on the unoccupied GR in vivo. However, the K_m was calculated from double-reciprocal analysis, which magnifies small variations in the experimental data. When these data were reanalyzed by direct fitting to the dose–response curve as discussed under Materials and Methods, there was no discrepancy between the EC_{50} and the K_m.

There were subtle differences between the effects of modulator on the GR vs the MR. Modulator 1 was 3–4-fold and modulator 2 was 2-fold more potent at stabilizing unoccupied MR steroid binding and inhibiting MR activation as compared to their effects on the GR. If the cellular concentration of the modulator isoforms is similar in liver and colon, both modulator isoforms might be more potent regulators of unoccupied steroid binding stability of the MR vs the GR. In addition, the maximum effect of modulator on stabilization of occupied receptor steroid binding was less for the MR than for the GR (maximum stabilization by modulator 1 was 44 ± 1 vs 109 ± 16% and by modulator 2 was 51 ± 9 vs 78 ± 6% for MR vs GR, respectively). This difference was not dose related, since the effect of modulator on the MR had plateaued at the highest concentrations tested. Finally, mixing of modulator isoforms had additive effects on all MR properties tested but was synergistic for inhibition of GR activation and stabilization of occupied GR steroid binding.

A model for the action of modulator on the GR and MR is shown in Figure 7. We postulate that modulator cross-links receptor and hsp-90, thereby maintaining the unactivated steroid-binding receptor form (Bodine & Litwack, 1990a,b). Confirmation that modulator binds directly to receptor will require synthesis of radiolabeled modulator, a project that our laboratory is currently pursuing. This model is based on the following observations: (1) modulator and molybdate appear to have identical effects on GR function (Bodine & Litwack, 1988b) and, as shown in this study, also have similar effects on the MR; (2) molybdate interacts directly with the GR protein (Bodine & Litwack, 1990a; Pratt et al., 1988) and the putative modulator/molybdate binding site on the rat GR and human MR share 90% conserved homology (Arriza et al., 1987; Bodine & Litwack, 1990a; Pratt et al., 1988); (3) formation of a complex between hsp-90 and GR maintains the unoccupied GR in a steroid-binding form (Dalman et al., 1989), and the unactivated MR also contains hsp-90; and (4)

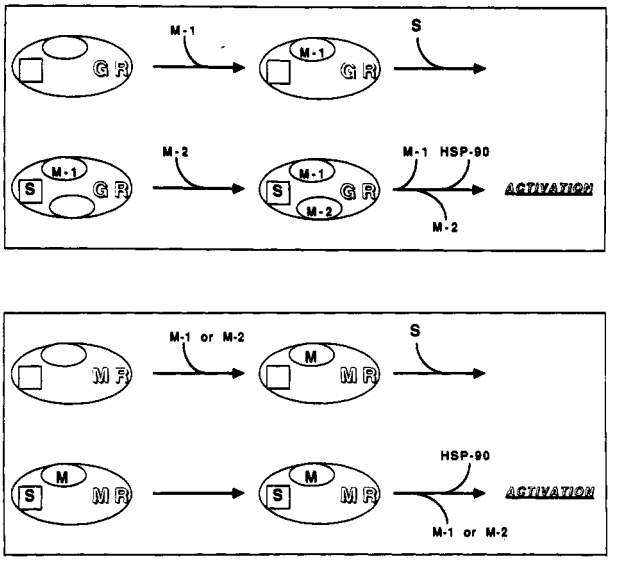

FIGURE 7: Hypothetical model for the interaction of modulator with the GR and MR. The top panel (GR) indicates that the unoccupied GR preferentially binds modulator 1 (M1) and that binding of steroid (S) exposes a binding site for modulator 2 (M2). M1 and M2 synergistically stabilize occupied GR steriod binding and inhibit receptor activation. The bottom panel (MR) indicates that the unoccupied and occupied MR both have one modulator binding site to which either M1 or M2 bind. Activation of both receptors requires dissociation of M and hsp-90 from the receptor. Further details are given in the text.

activation requires dissociation of GR from hsp-90 (Joab et al., 1984; Munck et al., 1990; Bodine & Litwack, 1990a). Since the effects of modulators on unoccupied GR steroid binding stability follow linear kinetics and are additive, we propose that the unoccupied GR has one modulator binding site that preferentially binds modulator 1 in vivo. The synergism of modulator 1 and 2 for occupied GR steroid binding stability and activation inhibition suggest that the occupied GR has two modulator binding sites which exhibit positive cooperativity or that both isoforms bind in a cooperative manner to a single site. The linear kinetics and additive effects of mixing modulator isoforms on the MR suggest that the unoccupied and occupied MR either have only one modulator binding site and/or two noncooperative binding sites. Since the EC_{50} and K_m of both modulator 1 and 2 for unoccupied MR steroid binding stability and activation inhibition are compatible with their estimated physiologic concentrations, both isoforms might be active in vivo. It is possible that the

observed lack of synergy between modulator isoforms might be an artifact of occupied MR proteolysis, resulting in the loss of a modulator binding site. If, as proposed, modulator must dissociate from the MR for activation to occur, then the modulator-binding site must be lost prior to activation. This possibility cannot be excluded until radiolabeled synthetic modulators become available. However, since most MR warmed in the presence of modulator had a R_s of 73 Å, MR appears to remain intact prior to activation.

Purified modulator regulates the function of two steroid hormone receptors, and modulator and molybdate have similar effects. Since molybdate stabilizes steroid binding and inhibits activation of other steroid receptors, these data suggest that modulator may be the endogenous factor that molybdate mimics. Future studies will determine if modulator also affects other steroid receptors.

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