Stabilization of 8S Progesterone Receptor from Human Prostate in the Presence of Molybdate Ion

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ABSTRACT

In the presence of 10 mM molybdate ion, we are able to detect the appearance of a [6,7-3H]-17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione-([3H]R5020) binding moiety in human prostatic cytosol which sedimented at approximately 8S in a glycerol density gradient. The specifically bound [3H]-R5020 was displaced by progesterone, tiramcinolone acetate, and R5020 but not by cortisol, dihydrotestosterone, 17β-estradiol, or diethylstilbestrol. Specific binding of [3H]R5020 in the presence of molybdate ion was shown to saturate at 7 x 10⁻¹⁰ M (n = 64 fmol/mg of protein). The optimum concentration of molybdate ion for enhancing specific binding of [3H]-R5020 was determined to be between 7 and 20 mM. Molybdate ion was also effective in stabilizing the 8S R5020-binding moiety in a preincubation for 16 hr at 0° in the absence of added steroid.

INTRODUCTION

Various investigators have established the presence of progesterone receptors in human prostate tissue (1-3, 6, 8, 13). Progestational agents such as cyproterone acetate have been used to treat benign prostatic hyperplasia and prostatic carcinoma with some success (7, 18, 28). The measurement of steroid receptor levels is very useful in predicting the response to endocrine therapy in patients with advanced breast cancer (19). There may be a similar relationship between steroid receptor levels and the responsiveness to endocrine therapy in prostatic cancer. Our laboratory has been interested in establishing an experimental protocol which would yield reliable estimates of progesterone receptor levels, since this receptor has been found to be quite labile (3).

In the papers of Nelson et al. (15) and Sando et al. (22, 23), it was reported that molybdate ion is effective in inhibiting the inactivation of glucocorticoid receptors from mouse fibroblasts, rat liver, and rat lymphocytes. We wish to report here the stabilizing effects that molybdate ion has on the progesterone receptor from human prostate.

MATERIALS AND METHODS

All water was glass distilled. All procedures were carried out at 4° unless otherwise noted. All pH values correspond to values at 0° as determined with a Corning No. 476050 combination electrode using Beckman No. 566002 buffer. All steroids were pipetted from freshly prepared aqueous ethanol solutions. Radioactivity was measured according to the procedure of Patterson and Green (17) using Triton X-100 and toluene in a Packard 2425 scintillation spectrometer system. Efficiencies were between 30 and 40%.

Sodium molybdate was purchased from Ventron Corp. (Denver, Mass.). [3H]R5020 was obtained New England Nuclear (Boston, Mass.; specific activity, 87 Ci/mmol). Radioactive bovine serum albumin and γ-globulin were prepared by acetylation of the unlabeled proteins with [14C]acetic anhydride according to the method of Silteri et al. (27). All other compounds were reagent grade chemicals obtained from Sigma Chemical Co. (Saint Louis, Mo.). The buffer system (TED buffer) used in this study had a pH adjusted to 7.4.

Fresh human prostate tissue was obtained from patients with benign prostatic hypertrophy by either transurethral resections or open prostatectomies which were performed at the University of Maryland Hospital. For experiments in which prostate tissue was to be frozen prior to homogenization, the tissue chips were immediately frozen in liquid nitrogen and stored at -70° in a deep freeze until use. The frozen chips were then pulverized and allowed to thaw just prior to homogenization. For experiments in which prostate tissue was to be used fresh, the tissue chips were finely minced with surgical scissors. Both frozen and fresh samples were handled identically from this stage of the procedure onward. The tissue was diluted (1 ml of wet tissue) with TED buffer which contained 10% (w/v) glycerol. The tissue suspension was then homogenized with a Polytron PT 10-35 homogenizer fitted with a PT 10 probe. Three 5-sec bursts of the Polytron (with 60-sec cooling periods between) were sufficient to homogenize the tissue. The homogenate was then centrifuged at 100,000 x g for 1 hr at 0° in a Beckman 50.1 rotor. After centrifugation, the protein concentration in the supernatant (cytosol) was estimated according to the procedure of Lowry et al. (12).

The cytosol was incubated with 8 x 10⁻⁹ M [3H]R5020 at 0-2° for 15 to 23 hr in TED buffer, containing 10% (w/v) glycerol and 0.8% ethanol. To determine the specificity of binding, parallel incubation tubes also contained 8 x 10⁻⁷ M quantities of unlabeled steroids. Incubation periods were terminated by adding an equal volume of a charcoal-coated dextran solution [dextran (0.005 g/100 ml), 0.5% Norit A, and 10% glycerol in TED buffer] to each tube. After incubation of this suspension for 15 min at 0°, the tubes were centrifuged at 3000 x g at 0° for 20 min. Aliquots were then removed for determination of the radioactivity or glycerol density gradients. Specific binding was defined as the amount of [3H]R5020 binding which could not be displaced by unlabeled steroids.
be displaced by an 100-fold excess of unlabeled R5020 or progesterone.

Linear 10 to 30% (w/v) glycerol density gradients (5 ml) in cellulose nitrate tubes were prepared in TED buffer. The linearity of the gradients was checked by the use of a Bausch and Lomb refractometer. Four-tenths ml of each incubated cytosol sample (which had been treated with dextran-coated charcoal) was layered onto the gradient. The sedimentation coefficients of the [3H]-steroid-labeled binding proteins were estimated by comparison with the sedimentation of a [14C]-bovine serum albumin and [14C]γ-globulin markers added to parallel gradients (approximately 9000 dpm in 5 μl buffer). Gradient tubes were centrifuged at 149,000 x g for 16 hr at 0° using a Beckman SW50.1 rotor. After centrifugation, the gradients were fractionated by inserting a thin steel tube from the top to the bottom of the tube and then removing 3-drop (approximately 0.2-ml) fractions with a Technician peristaltic pump directly into liquid scintillation vials.

For Scatchard (24) analysis, cytosol was incubated with [3H]R5020 (8.4 x 10^-11 M to 2 x 10^-9 M) for 20 hr at 0° in the presence and absence of unlabeled R5020 (2 x 10^-7 M). In all tubes, the final ethanol concentration was 1.1%, and the molybdate concentration was 10 mM. At the end of the incubation period, the radioactivity of the samples (total) was determined in duplicate 10-μl portions. The free steroid was then adsorbed from solution by the addition of 200 μl of dextran-coated charcoal solution. The charcoal-cytosol suspension was incubated for 15 min at 0°. The samples were then centrifuged at 3000 x g at 0° for 20 min. The radioactivity of the supernatant (bound) was then determined in duplicate 100-μl samples. The plots were corrected for nonsaturable binding as described by Chamness and McGuire (4). The concentration of free steroid in each sample was estimated by the difference of total and bound radioactivity.

RESULTS

The effects of molybdate ion on the binding of [3H]R5020 to prostate cytosol were examined by glycerol density gradient centrifugation. Prostatic cytosol samples were incubated with [3H]R5020 in the absence or presence of 10 mM sodium molybdate. The sedimentation patterns of prostatic cytosol incubated in the absence of added molybdate shows a distinct peak of bound [3H]R5020 (Chart 1A, □) which is characterized by a sedimentation coefficient of approximately 4S. The specificity of binding was demonstrated by the displacement of [3H]-R5020 binding in the 4S region when 100-fold excess of unlabeled progesterone was present in the incubation mixture (Chart 1A, C). Chart 1B demonstrates the sedimentation patterns of prostatic cytosol that was incubated with [3H]R5020 in the presence of 10 mM molybdate. It can be seen that there are 2 distinct peaks of bound [3H]R5020 (Chart 1B, ◦) characterized by sedimentation coefficients of approximately 8S and approximately 4S. Furthermore, only the binding of [3H]-R5020 in the 8S region was displaced by the presence of 100-fold excess of unlabeled progesterone (Chart 1B, C). Moreover, when lower concentrations of hormone (2 or 4 x 10^-9 M [3H]R5020) were incubated with prostatic cytosol again in the presence of molybdate ion, the specific binding of [3H]-R5020 sedimented also at 8S region.4 These results suggest that in the presence of 10 mM molybdate ion the specific binding of [3H]R5020 sediments as an 8S component. This binding would otherwise (in the absence of molybdate) sediment as a 4S component.

In order to further investigate the effect of molybdate ion on specific [3H]R5020 binding, prostatic cytosol was preincubated for 16 hr at 0° in the absence of 10 mM added molybdate. At the end of this time, appropriate steroid solutions were added to each of the parallel cytosol mixtures. Chart 2A shows that, in the absence of added molybdate, little or no specific binding of [3H]R5020 could be demonstrated. Chart 2B shows that prostatic cytosol preincubated in the presence

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4 N. Bashiri et al., unpublished results.

**Chart 1.** Glycerol density gradient sedimentation patterns of human prostatic cytosol (tissue was obtained by transurethral resection and then frozen) incubated with 8 x 10^-4 M [3H]R5020 for 15 hr at 0-2°C, in the absence (A) or presence (B) of 7 x 10^-7 M progesterone for 24 hr at 0-2°C. Total specific binding was 10.0 fmol/mg of protein in A and 10.7 fmol/mg of protein in B.

**Chart 2.** Glycerol density gradient sedimentation patterns of human prostatic cytosol (same tissue sample which was used for experiments in Chart 1) preincubated in the absence of added steroid for 16 hr at 0-2°C in the absence (A) or presence (B) of 10 mM sodium molybdate. The incubation mixtures contained, in a final volume of 500 μl: cytosol (6 mg of protein per ml) in TED buffer (pH 7.4) containing 10% (w/v) glycerol; 0.8% ethanol; and either 8 x 10^-4 M [3H]R5020 alone (A) or 8 x 10^-4 M [3H]R5020 and 8 x 10^-7 M unlabeled progesterone (B). Total specific binding was 4.7 fmol/mg of protein in A and 11.8 fmol/mg of protein in B.
of 10 mM molybdate ion contains [3H]R5020-binding components which sedimented in both 8S and 4S regions of the gradients. Furthermore, the binding of [3H]R5020 in the 8S region was displaced by the presence of 100-fold excess of unlabeled progesterone, indicating that the specific binding lies almost entirely in the 8S region.

To demonstrate that the effects of molybdate on the specific binding of [3H]R5020 were not due solely to ionic strength effects, cytosol was incubated with [3H]R5020 at 0° for 18 to 20 hr in the presence of 10 mM NaCl, 10 mM Na2SO4, and 10 mM Na2MoO4, and also in the absence of added salt. Chart 3 demonstrates that the presence of either 10 mM chloride (●) or 10 mM sulfate (△) had little effect on sedimentation patterns of [3H]R5020 binding which was characterized by a peak in the 4S region. On the other hand, sedimentation patterns of cytosol incubated in the presence of 10 mM molybdate (●) demonstrate [3H]R5020 binding in both 4S and 8S regions. The binding in the 8S region was displaced with 100-fold excess unlabeled R5020 (x) or unlabeled progesterone (△). We have also demonstrated by single-point assays that the presence of 5, 10, or 25 mM added sodium sulfate or sodium chloride cause, if anything, a reduction of specific [3H]R5020 binding (data not presented).

In order to investigate the steroid specificity of the [3H]-R5020-binding components, cytosol was incubated with 10 mM molybdate and 1 × 10−9 M [3H]R5020 in the absence and presence of a 10-, 100-, 1000-fold excess of nonradioactive steroids. Nonspecific binding, defined as that binding not displaced by the addition of a 100-fold excess of nonradioactive R5020 or progesterone, was subtracted in all instances. The amount of [3H]R5020 bound in the presence of each concentration of nonradioactive steroid was expressed as a percentage with respect to the amount of [3H]R5020 bound in the absence of any nonradioactive steroid. The results are presented in Chart 4. It can be seen that progesterone and triamcinolone acetonide [which specifically binds to the progesterone receptor (29)] are as effective as R5020 in competing for [3H]R5020-binding sites. Cortisol, dihydrotestosterone and estradiol compete poorly for [3H]R5020 binding even at concentration levels 1000-fold in excess of the radioligand. Diethylstilbestrol does not compete at any concentration tested.

A saturation curve for the binding of [3H]R5020 to cytosol in the presence of 10 mM molybdate is shown in Chart 5A. Specific binding was determined by subtracting the number of moles of [3H]R5020 bound in presence of 100-fold excess of...
RS5020 from the number of moles bound in the absence of added unlabeled steroid. From these data, a dissociation constant of $3.5 \times 10^{-10}$ M was estimated. Chart 5B demonstrates the Scatchard analysis of the data presented in Chart 5A corrected for nonspecific binding by the method of Chamness and McGuire (4, 24). Data from Chart 5B indicated a corrected dissociation constant of $3.7 \times 10^{-10}$ M and a corrected number of apparent binding sites of 64 fmol/mg of protein.

In order to demonstrate that the effects of molybdate were concentration dependent, cytosol samples were incubated in the presence of various concentrations of molybdate ion. Chart 6 shows that the highest levels of specific binding of $[^3H]RS5020$ are achieved in the presence approximately 7 to 20 mM added molybdate.

**DISCUSSION**

In general, about 50 to 70% of tumors that are estrogen receptor-positive can be expected to respond objectively to endocrine therapy, whereas about 5 to 10% of receptor-negative tumors will respond (11). One explanation for the response of the hormone receptor-negative patients is that the steroid receptors are thermolabile and are unstable proteins (11).

Measuring progesterone receptor in human breast cancer biopsies, McGuire et al. (19) have concluded that the 8S component of sucrose density gradient profiles yields the most reliable data on progesterone receptor concentrations in human breast tumors. Because progesterone receptor in human prostatic cytosol has been shown to be so unstable, efforts were made to find means to stabilize this receptor. In the presence of 10 mM molybdate ion, we were able to detect the appearance of a binding moiety which sedimented at approximately 8S in a glycerol density gradient. Such an 8S R5020-binding moiety has been reported in the absence of molybdate (3). However, we were able to demonstrate in this report that molybdate ion caused the appearance of an 8S peak from several individual cytosol preparations which, under identical conditions except in the absence of added molybdate ion, did not show such an 8S binding moiety. Molybdate ion was effective in stabilizing the 8S R5020-binding moiety in a precubation for 16 hr at 0° in the absence of added steroid. These results are consistent with the conclusion that molybdate ion...

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*Chart 4.* Prostatic cytosol samples (obtained by transurethral resection and used fresh; 3.6 mg of protein per ml) containing 10 mM sodium molybdate were incubated with $1 \times 10^{-8}$ M $[^3H]RS5020$ in the absence or presence of 10-, 100-, or 1000-fold excess of competitors at 0-2° for 23 hr. Nonspecific binding, defined as bound $[^3H]RS5020$ cpm which were not displaced by the addition of 100-fold excess of cold RS5020, were subtracted in all instances. DES, diethylstilbestrol; DHT, dihydrotestosterone.

*Chart 5.* Prostatic cytosol samples (obtained by retropubic prostatectomy and were then frozen; 1.5 mg, protein per ml) containing 10 mM sodium molybdate were incubated for 18 hr at 0-2° with $8.4 \times 10^{-11}$ to $2 \times 10^{-8}$ M $[^3H]RS5020$ in the absence (A, ○) and in the presence (A, ×) of $2 \times 10^{-8}$ M RS5020. Specific binding (A, ×) was generated by subtracting nonspecific from total binding. Scatchard analysis (B, ●) of $[^3H]RS5020$ binding to prostatic cytosol in the presence of 10 mM molybdate ion was generated from data in A. The plot is corrected for nonsaturated binding (○) as described by Chamness and McGuire (4). B, bound; F, free.
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Chart 6. Prostatic cytosol samples (obtained by retropubic prostatectomy and were then frozen; 3.4 mg of protein per ml) were incubated with $4 \times 10^{-9}$ M $[^3H]R5020$ in the presence of $1 \times 10^{-7}$ M to $7 \times 10^{-8}$ M sodium molybdate for 21 hr at 0–2°C. In addition, for each concentration of sodium molybdate, prostatic cytosol samples were incubated with $4 \times 10^{-9}$ M $[^3H]R5020$ and $4 \times 10^{-17}$ M progesterone to determine nonspecific binding. The data presented represent total specific binding. Control samples containing no added molybdate yielded 350 cpm of specifically bound $[^3H]R5020$.

has a stabilizing effect on the 8S form of the progesterone receptor.

The mechanism by which molybdate ion stabilizes the 8S form of the receptor may also be of theoretical interest. Three possible hypotheses, which we are using as a basis for further investigation, are worth mentioning. Molybdate ion is known to inhibit phosphatase activity in in vitro preparations from both plant and animal sources (5, 9, 16, 21). Pratt et al. have concluded from their work with glucocorticoid receptor preparations from mouse fibroblasts (14, 23), rat liver (15), and rat lymphocytes (22) that dephosphorylation may play a role in the temperature-dependent loss of glucocorticoid receptor binding capacity. On the basis of the results of experiments using molybdate ion and ATP, these investigators postulated that the binding capacity of the glucocorticoid receptor was dependent upon a phosphorylation step. Similarly, molybdate ion may act as a phosphatase inhibitor in our system; as a result, it may allow the receptor to remain in a phosphorylated state. The presence of a phosphate group on the receptor may then ultimately affect the stability and sedimentation value of the receptor. However, there is no evidence of a phosphorylated amino acid from characterization studies of purified progesterone receptor from chick oviduct (10, 25, 26).

Alternatively, molybdate ion may affect the activity of another enzyme, such as adenylate cyclase, which could then lead to a change in the progesterone-binding capacity of the cytosol preparation. A stimulation of in vitro adenylate cyclase activity from rat liver plasma membranes by molybdate ion has recently been shown by Richards and Swislocki (20).

A third possible mechanism for the effects of molybdate would not involve enzyme activity at all. Rather, molybdate may exert its effect via charge stabilization. Thus, in our system, molybdate ion (or a molybdate complex) may stabilize positive charges on the 8S form of the progesterone receptor.

Current research activities in our laboratory are focused to elucidate the mechanism by which molybdate ion stabilizes the 8S form of the progesterone receptor.

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