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SPECIFICITY OF MEDROXYPROGESTERONE ACETATE BINDING IN HUMAN ENDOMETRIUM: INTERACTION WITH TESTOSTERONE AND PROGESTERONE BINDING SITES

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SUMMARY

Human endometrial cytosol contains high affinity, low capacity binder(s) for testosterone as well as for progesterone and medroxyprogesterone acetate (MPA). The equilibrium constant of dissociation is the same for all three compounds. In a series of individual samples the concentration of binding sites for [^{3}H]-MPA did not exhibit the cyclic variations of the progesterone binder and was consistently more than 3 × higher. The latter observation was confirmed when [^{3}H]-progesterone and [^{3}H]-MPA binding were directly compared using several different pools of endometrial cytosol. Competition studies employing cytosol pools demonstrated that, unlike [^{3}H]-progesterone, [^{3}H]-MPA is displaced from its binding sites by an excess of unlabeled testosterone. Furthermore, [^{3}H]-MPA and [^{3}H]-testosterone binding were demonstrable in a cytosol pool that contained no detectable binder for [^{3}H]-progesterone. It was concluded that while testosterone and progesterone each interact chiefly with their specific binders, MPA has broader specificity and interacts with binders for both of the other steroids. Unless this property of dual binding is recognized and measured, values for "progesterone-specific" binding sites determined using MPA as the test progestin will be greatly overestimated.

INTRODUCTION

Normal human endometrial [1–14] and myometrial [3, 7, 14–22] cytosols contain a binder of progesterone that has properties similar to those of a steroid receptor. The concentration but apparently not the affinity constant of this binder varies with the stage of the menstrual cycle [2, 5, 8–11, 18–20]. It is clear that, although cytosol samples of these tissues are usually contaminated with plasma proteins, the tissue binder is different from the high affinity plasma binders of progesterone. Several techniques have been employed to prevent plasma binders, corticosteroid binding globulin (CBG) for example, from interfering with progesterone binding determinations on cytosols. Unlabeled cortisol has been successfully used to saturate CBG binding sites [4–6, 10, 20]. Synthetic compounds with progestational activity have little or no affinity for plasma steroid binders and have been used as radiolabeled ligands [7, 12–14, 19] or as unlabeled competitors [5, 18, 19]. Data derived from the use of such compounds, however, must be validated by establishing that they interact solely with progesterone-specific sites and not with binders for other steroids. Medroxyprogesterone acetate (MPA, 17-hydroxy-6α-methylpregn-4-ene-3,20-dione 17-acetate), for example, a compound widely used for the treatment of endometrial hyperplasia and carcinoma [23], competes directly for androgen-specific high affinity binding sites in mouse kidney [24] and has glucocorticoid activity *in vivo* in humans [25]. These studies, designed to test the specificity of the binding of MPA in human endometrial cytosols and the potential use of this compound as a measure of progesterone-specific binding, required testing for the presence of an androgen binder in this tissue. Glucocorticoid-specific binding had been sought in our previous studies and was not detectable [10].

MATERIAL AND METHODS

Chemicals. All [^{3}H]-labeled steroids were purchased from New England Nuclear Company, Boston, Massachusetts. The specific activity of each steroid preparation is listed in the legends of the figures. Unlabeled steroids were obtained from Mann Research Laboratories, New York, NY, and Sigma Chemical Company, St. Louis, Missouri. Norit used for the separation of bound and free steroids was purchased from Fisher Scientific Company, Fairlawn, New Jersey, and the dextran from Pharmacia. Aquasol II for liquid scintillation counting was obtained from New England Nuclear. All other reagents and chemicals were obtained in the purest grade available.

Measurement of radioactivity. Tritium-labeled samples were counted in Aquasol II in a Searle-Nuclear Chicago Mark II liquid scintillation spectro-

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photometer at an average efficiency of 30%. Data were evaluated as described elsewhere [10].

Analysis of data. Binding data for these studies were analyzed in the same manner as in our previous studies [10].

Histological evaluation of endometrium. All endometrial samples were dated histologically according to the criteria of Noyes *et al.*[26]. The same pathologists reviewed all of the specimens used in this study without knowledge of the results of the steroid binding studies [10].

Preparation of cytosols. Fresh endometrial samples obtained by curettage were homogenized and centrifuged as previously described [10]. The average wet weight of these specimens was 514 mg ± 84 (S.E.M.) and the average protein concentration of resulting cytosols was 4.5 ± 0.7 mg/ml (S.E.M.) as determined by the method of Lowry *et al.*[27].

Steroid binding assay. The determination of radiolabeled steroid binding by endometrial cytosols, individual specimens or pools, was carried out as previously described [10] with slight variations. The cytosols were kept at 4°C through all steps of the procedure. Cytosol, adjusted to 1 mg/ml protein, 0.25 ml/tube, was incubated with gentle shaking for 4 h with increasing concentrations of tritiated steroid in the presence or absence of a constant molar excess of the same steroid without tritium. Bound and free steroid were separated by treatment with dextran-coated charcoal for 10–30 min followed by centrifugation. The concentration of labeled steroid bound in the presence of excess unlabeled steroid (non-displaceable binding) was subtracted from the amount bound in its absence (total binding). This difference, the displaceable binding, was recorded as specific binding and was plotted by a variation of the Scatchard method [28].

Competition studies. The experimental design was as described above except that the steroid used in excess in unlabeled form was the competing steroid. Again, the displaceable binding, now "specific" only in a figurative sense, was plotted.

Thin-layer chromatography of medroxyprogesterone acetate. A pool of endometrial cytosols was diluted to 1.0 mg protein/ml and 0.2 ml was incubated in quadruplicate for 4 h at 4°C with shaking with 1 nM [1,2-^{3}H]-MPA, 58 Ci/mmol. The incubation mixtures were extracted with 5.0 ml of methylene chloride and the organic phases were pooled and dried. Unlabeled MPA, 50 μg, was added to the dried extract, which was transferred in 0.1 ml methylene chloride to silica gel G-coated glass plates (EM Laboratories) and chromatographed 2× in cyclohexane–ethyl acetate (1:1, V/V). As a control, stock [^{3}H]-MPA diluted with unlabeled MPA, 50 μg was run from a different origin on the same plate. The MPA spot was located by ultraviolet absorption; its R_F was approximately 0.5. The chromatographic track was divided into 11 equal squares, including the origin and the MPA control spot, which were scraped from the plate and individually extracted by vortexing with 3 × 1 ml of chloroform–methanol (1:1, V/V). The extract was pooled in scintillator vials, dried, and counted for radioactivity.

Protamine sulfate precipitation. All steps were carried out at 4°C. Cytosols or sera were diluted to approximately 1 mg/protein/ml, aliquoted into 0.2 ml portions and the protein precipitated by the addition of protamine sulfate to a final concentration of 3.5 mg/ml. The mixtures were incubated for 20 min at 4°C, and then centrifuged for 10 min at 800 *g*. The supernatants were discarded and the pellets washed 3× with cold Tris–EDTA buffer (10 mM, 1.5 mM, pH 8). Tritiated steroid with or without unlabeled competitor was added in buffer to all tubes. The final incubation volume was 0.25 ml. The pellets were incubated for 4 h with shaking and centrifuged for 10 min at 800 *g*. The resulting supernatants were decanted and the pellets washed 3× with cold Tris–EDTA buffer (0.5 ml). The pellets were extracted with 3 × 1 ml volumes of ethanol with vortexing and Aquasol II (2 ml) was added to the dried alcohol extracts for counting.

RESULTS

MPA binding by endometrial cytosols

A representative Scatchard plot (Fig. 1) of specific (MPA-displaceable) binding of [^{3}H]-MPA by an individual endometrial cytosol demonstrates that the binding is of high affinity. The dissociation constant of 3.3 × 10^{-10} is similar to those reported for progesterone[10].

Control experiments measuring [^{3}H]-MPA binding

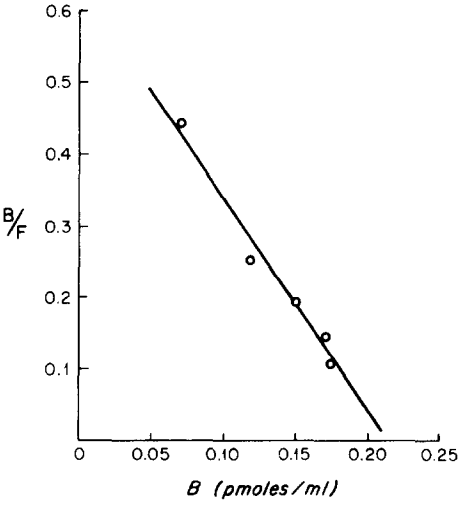

Fig. 1. Determination of the dissociation constant of [^{3}H]-MPA binding in a normal proliferative endometrial cytosol. Incubation was for 4 h at 4°C with shaking in the presence of [1,2-^{3}H]-MPA (58 Ci/mmol) and 1 μM unlabeled cortisol with and without a 250-fold molar excess of unlabeled MPA. Bound and free [^{3}H]-MPA were separated by dextran-coated charcoal. Plot of specifically bound MPA (B) vs. the ratio of bound to free (B/F). Each point is the average of duplicate determinations.

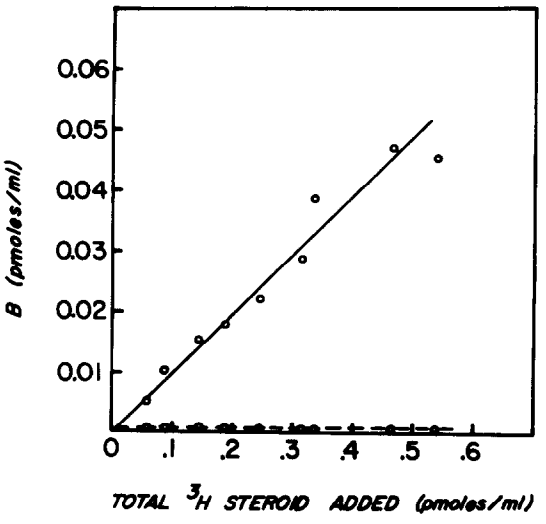

Fig. 2. [^{3}H]-MPA binding in human female serum diluted to 1 mg protein/ml with Tris–EDTA buffer (10 mM, 1.5 mM, pH 8). Diluted serum was incubated for 4 h at 4°C with increasing concentrations of [1,2-^{3}H]-MPA (58 Ci/mmol) in duplicate in the presence or absence of a 250-fold molar excess of unlabeled MPA. Bound and free [^{3}H]-MPA were separated by dextran-coated charcoal. The solid line is total binding and the dashed line represents specific [^{3}H]-MPA binding.

to human female serum diluted to 1 mg/ml protein demonstrate the lack of specific binding of [^{3}H]-MPA (Fig. 2). The total binding of [^{3}H]-MPA to serum increases with increasing [^{3}H]-steroid added but none of this binding is blocked by a large molar excess of unlabeled MPA. It is unlikely, therefore, that the specific binding of [^{3}H]-MPA observed in endometrial cytosols is due to serum binders contaminating the preparations.

Furthermore, [^{3}H]-MPA recovered from 4 h incubations with endometrial cytosol remained over 97% pure and free of minor radioactive peaks on thin layer chromatography, ruling out the possibility that the bound radioactivity might be a metabolite of MPA rather than MPA itself (see Materials and Methods).

Concentration of MPA binding sites in the menstrual cycle

Thirty endometrial samples, dated histologically by members of the pathology department, were tested for the presence of high affinity specific [^{3}H]-MPA binding. The concentration of binding sites was estimated from the Scatchard plots and recorded as fmol/mg of cytosol protein or fmol/mg of tissue. Unlike [^{3}H]-progesterone binding [2, 5, 8–11] the average concentration of [^{3}H]-MPA binding sites in the proliferative phase was not significantly higher than that observed in the secretory phase. Also, the average value of [^{3}H]-MPA binding throughout the cycle (350 ± 260 S.E.M. fmol/mg protein) was significantly higher ($P < 0.05$) than the values previously obtained with [^{3}H]-progesterone (average 65 ± 26 fmol/mg protein) in this laboratory [10].

Direct comparison of MPA and progesterone binding in pooled endometrial cytosol

The foregoing results could have been caused by some difference between current and earlier assay procedures or between current endometrial samples and those obtained earlier. In order to rule out these considerations, the binding of [^{3}H]-MPA and [^{3}H]-progesterone was measured in the same cytosol preparation. In order to have enough material for these studies it was necessary to form pools of endometrial cytosols. This was done by mixing 4–6 cytosols to a volume of about 20 ml containing 4 mg/ml of protein. The specific binding of [^{3}H]-MPA and [^{3}H]-progesterone in such a pool proved to be saturable when increasing concentrations of labeled steroids were incubated with a constant amount of the cytosol pool, but [^{3}H]-MPA binding was higher than that observed for [^{3}H]-progesterone over a similar dose range of steroid added. Figure 3 shows the Scatchard plots of the specific binding data. The slopes of the linear portion of the two curves are nearly parallel indicating similar dissociation constants (1.5×10^{-10} M). The binding curves for both steroids become almost horizontal at approximately the same B/F ratio, indicating the presence of about the same proportion of low-affinity but nonetheless specific (displaceable) binder for both steroids. The intercepts with the abscissa, however, are different, confirming the presence of a greater concentration of high-affinity binding sites for [^{3}H]-MPA than for [^{3}H]-progesterone. Results obtained in pools support the observation made on individual samples: the concentration of binding sites is greater for [^{3}H]-MPA than for [^{3}H]-progesterone, while the binding constants are the same.

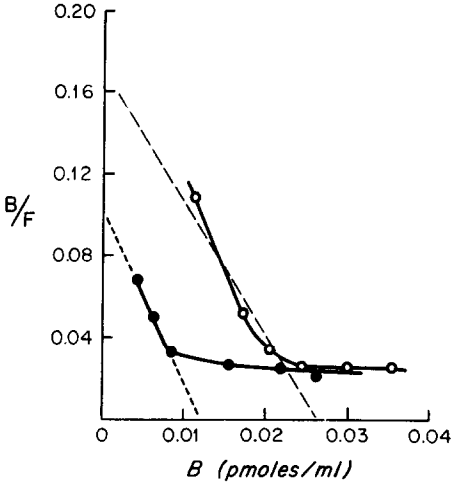

Fig. 3. B/F vs. B plot of specific binding of [^{3}H]-progesterone and [^{3}H]-MPA as determined by dextran-coated charcoal assays. Progesterone-specific (●) and MPA-specific binding (○) are shown. Dashed lines represent slopes calculated by the method of least squares.

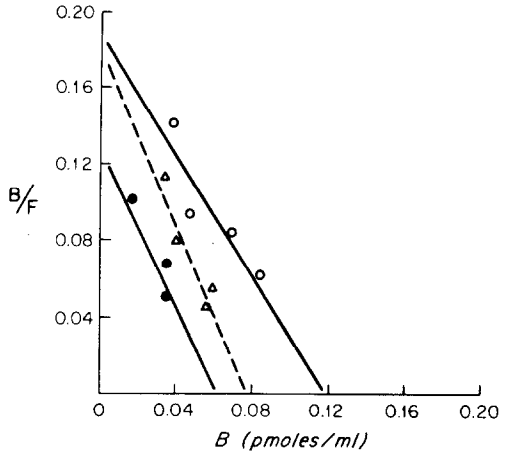

Fig. 4. B/F vs. B plot of [^{3}H]-progesterone and [^{3}H]-MPA binding in competition experiments. Bound and free [^{3}H]-steroids were separated by dextran-coated charcoal. Progesterone-specific binding (●—●), MPA-specific binding (○—○) and testosterone-displaceable MPA binding (△—△) are shown; slopes are derived from least squares calculations. Cytosol pooled from normal proliferative and secretory endometria adjusted to ~1 mg/ml protein was incubated for 4 h at 4°C with shaking. The excess of unlabeled hormone was 250-fold in each case, and [1,2,6,7-^{3}H]-progesterone (107 Ci/mmol) and [1,2-^{3}H]-MPA (58 Ci/mmol) were used as labeled ligands.

Competition studies on MPA and progesterone binding by pools of endometrial cytosols

Constant amounts of cytosol pool were incubated with increasing concentrations of tritiated MPA or progesterone in the absence or presence of a constant molar excess of unlabeled MPA, progesterone, testosterone, or dexamethasone. No significant displacement of [^{3}H]-MPA or [^{3}H]-progesterone binding was observed when dexamethasone was used as a competitor (data not shown). The Scatchard plots obtained from this experiment are shown in Fig. 4 which is a composite of [^{3}H]-MPA-specific and [^{3}H]-progesterone-specific binding as well as testosterone-displaceable [^{3}H]-MPA binding. In this pool the dissociation constants of [^{3}H]-MPA and [^{3}H]-progesterone-specific binding are 6×10^{-10} M and 5×10^{-10} M respectively. Here, as before, there is a greater number of [^{3}H]-MPA binding sites than those detected for [^{3}H]-progesterone. Testosterone displaces little [^{3}H]-progesterone binding under these conditions and the B/F vs. B plot of testosterone-displaceable [^{3}H]-progesterone binding does not have a statistically significant slope (data not shown). Testosterone-displaceable [^{3}H]-MPA binding, however, is considerable and reflects interaction with high affinity binding sites. The slope of the Scatchard-type plot of testosterone-displaceable [^{3}H]-MPA binding is nearly equal to the slopes of [^{3}H]-MPA- and [^{3}H]-progesterone-specific binding. In addition, the sum of the concentration of binding sites for [^{3}H]-progesterone-specific and testosterone-displaceable [^{3}H]-MPA binding sites approximates the value for [^{3}H]-MPA-specific sites in this pool. Testosterone, therefore, shows little interference with [^{3}H]-progesterone binding, while it competes effectively for some, but not all, of the [^{3}H]-MPA binding sites. Dexamethasone, on the other hand, has no effect on [^{3}H]-MPA or [^{3}H]-progesterone binding. It is possible that [^{3}H]-MPA is binding to sites in human endometrium that are unavailable to [^{3}H]-progesterone but are capable of interacting with testosterone.

Binding of [^{3}H]-testosterone in the absence of [^{3}H]-progesterone binding

In order to test the possibility that MPA is interacting at least in part with testosterone binding sites in endometrial cytosols it was necessary to demonstrate testosterone-specific binding of high affinity in this tissue. A pool of proliferative and secretory endometrial cytosols was incubated with increasing concentrations of [^{3}H]-MPA, [^{3}H]-progesterone or [^{3}H]-testosterone in the presence or absence of an excess of the corresponding unlabeled steroid. This pool, unlike the previous preparation, bound [^{3}H]-MPA but not [^{3}H]-progesterone specifically. The Scatchard plots for [^{3}H]-MPA- and [^{3}H]-testosterone-binding by this pool seen in Fig. 5 show high affinity specific binding with dissociation constants of 5×10^{-10} M and 10×10^{-10} M respectively.

SHBG is not protamine-precipitable

Protamine sulfate precipitation was tested as a means of separating cytosol binders from serum sex hormone binding globulin (SHBG). Tritiated testosterone binding by diluted human female serum (protein 1 mg/ml) was demonstrable using the dextran-coated charcoal assay (Fig. 6, panel A), but the binding protein was not precipitable by protamine sulfate (Fig. 6, panel B).

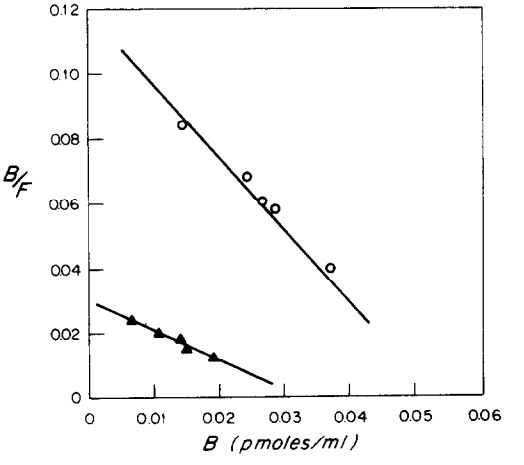

Fig. 5. B/F vs. B plot for specifically bound [^{3}H]-MPA (○), and [^{3}H]-testosterone (▲). Specific binding of [^{3}H]-progesterone was zero for this pool of endometrial cytosols.

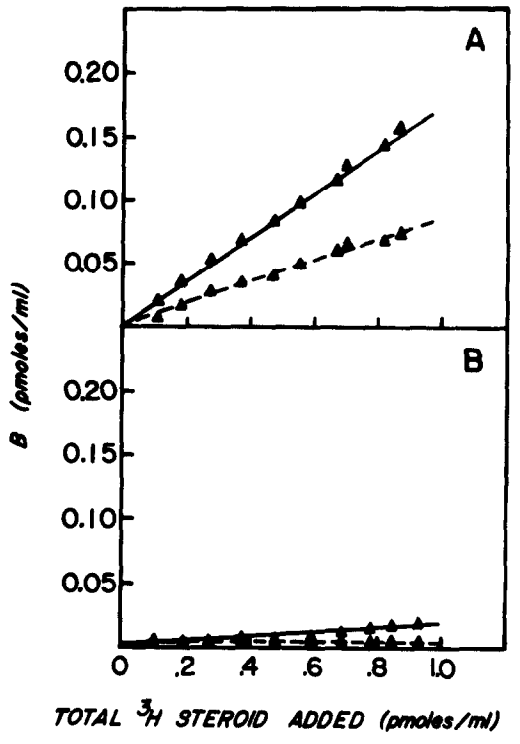

Fig. 6. *Panel A.* [^{3}H]-Testosterone binding in human female serum diluted to 1 mg protein/ml with Tris–EDTA buffer (10 mM, 1.5 mM, pH 8). Diluted serum 200 μl was incubated for 4 h at 4°C with increasing concentrations of [1,2,6,7-^{3}H]-testosterone (85 Ci/mmol) in duplicate in the presence or absence of a 250-fold molar excess of unlabeled testosterone. Final incubation volume was 0.25 mls. Bound and free [^{3}H]-testosterone were separated by the addition of dextran-coated charcoal (see Materials and Methods). Total binding, solid lines, or specific binding, dashed lines, are shown. *Panel B.* [^{3}H]-Testosterone binding in the diluted human female serum described in Panel A as determined following precipitation of the serum by protamine sulfate (see Materials and Methods). The serum pellets were incubated for 4 h at 4°C with increasing concentrations of [1,2,6,7-^{3}H]-testosterone (85 Ci/mmol) in duplicate in the presence or absence of a 250-fold molar excess of unlabeled testosterone. Total binding, solid lines, and specific binding, dashed lines, are shown.

Specificities of protamine-precipitated cytosolic binders

Using the same experimental protocol a third cytosol pool was precipitated with protamine sulfate and the pellets were incubated with either [^{3}H]-MPA, [^{3}H]-progesterone or [^{3}H]-testosterone with and without unlabeled MPA, progesterone or testosterone for 4 h at 4°C. This pool exhibited specific binding for all three steroids. The displacement of [^{3}H]-progesterone binding in the precipitated cytosol pool by unlabeled progesterone was normalized to 100% and the displacement by unlabeled MPA and testosterone compared to that value. Similar calculations were made when radiolabeled MPA or testosterone were used. The results of these experiments are shown in Fig. 7. Unlabeled MPA and unlabeled progesterone displace 70–80% of the specific binding sites for tritiated progesterone, MPA and testosterone. Unlabeled testosterone, however, displaces 40% of [^{3}H]-MPA binding but only 7% of [^{3}H]-progesterone binding.

DISCUSSION

Previous studies have shown that the high-affinity progesterone binder of endometrial cytosol varies in quantity but not affinity during the menstrual cycle, being maximal in the late proliferative phase [2, 5, 8–11]. Progesterone binding is not significantly displaced by androgens, estrogens or glucocorticoids but is quantitatively displaced by synthetic progestins. Since synthetic progestins such as MPA do not bind to high-affinity plasma components such as corticosteroid binding globulin (CBG) (Fig. 2) they are, potentially, specific probes for the tissue progesterone receptor. The present study was undertaken to test the affinity, specificity, and hence the usefulness of [^{3}H]-MPA for this purpose. If [^{3}H]-MPA exhibited a higher affinity for the binder than progesterone itself, it might be useful for this reason alone. This, however, is not the case: the dissociation constants of the two compounds are the same (Figs 1, 3 and 4). This also means that the larger concentration of sites detected by [^{3}H]-MPA (Figs 3 and 4) cannot be attributed to a more stable interaction with the binder, and implies that the additional sites are not progesterone-specific. MPA has glucocorticoid activity *in vivo* in man [25], but interaction with glucocorticoid sites in human endometrium are ruled out by the absence of glucocorticoid (dexamethasone) binder in human endometrium [10] and by the failure of unlabeled dexamethasone to displace [^{3}H]-MPA or [^{3}H]-progesterone. MPA has androgenic activity and interacts directly with androgen binding protein in mouse kidney [24]. The present data confirm the fact that testosterone does not compete for [^{3}H]-progesterone sites [10], and shows that it does complete significantly with [^{3}H]-MPA (Figs 4 and 7). High affinity [^{3}H]-testosterone-specific binding was demonstrated in endometrial cytosol. In one cytosol pool there was no [^{3}H]-progesterone-specific binding, but both testosterone- and MPA-specific binding were present (Fig. 5), thus making a clear separation between the progesterone and androgen binding sites that interact with MPA.

Protamine sulfate precipitation was used to rule out the possibility that testosterone binding might be due to contamination of the cytosol with SHBG (Fig. 6). Experiments performed on the cytosol pool precipitates again demonstrated [^{3}H]-testosterone binding and the displacement of [^{3}H]-MPA, but not of [^{3}H]-progesterone binding, by testosterone. Both MPA and progesterone competed for the [^{3}H]-testosterone binding sites (Fig. 7). The competition of progesterone for testosterone binding sites is paradoxical since testosterone is incapable of displacing progesterone, as noted above.

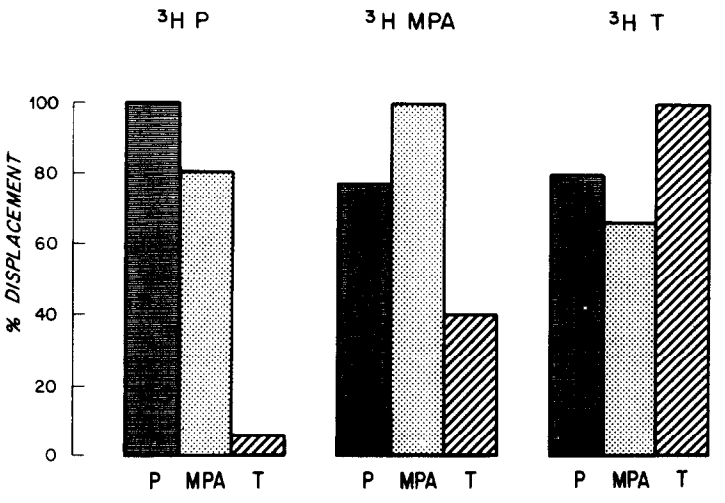

Fig. 7. Displacement of [^{3}H]-P ([1,2,6,7-^{3}H]-progesterone, 107 Ci/mmol), [^{3}H]-PA ([1,2-^{3}H]-MPA, 58 Ci/mmol) and [^{3}H]-T ([1,2,6,7-^{3}H]-testosterone, 85 Ci/mmol) by a 500-fold molar excess of unlabeled progesterone (P), MPA, or testosterone (T). Experiments were performed on protamine sulfate pellets of pooled proliferative and secretory endometrial cytosols adjusted to ~1 mg/ml protein concentration before precipitation.

The present study is a special case of a general problem with respect to the use of synthetic steroids as probes for physiological receptors. In this instance, the synthetic steroid [^{3}H]-MPA binds both to progesterone- and testosterone-specific binding sites, if not others, so that the amount bound cannot serve as a useful estimate of the number of sites for the other steroids. The displacement of testosterone by progesterone involves a small number of sites of unknown affinity and possibly of low specificity with respect to either steroid. The fact that testosterone cannot displace progesterone implies a high degree of specificity for the progesterone binding sites as compared to MPA binding sites. Nonetheless, an accurate picture of progesterone receptor-like binding in human endometrium is obtained by using [^{3}H]-progesterone itself and controlling for CBG by adding unlabeled cortisol, or by precipitation with protamine sulfate. It follows that when a synthetic steroid is used to estimate the binding capacity of a tissue for a physiological steroid, other receptors that may interact with it must be removed or saturated with their respective ligands.

Androgen binding has been reported in rat [29–30] and human uterus [31–32], and this report confirms the observation in human endometrium. The role of an androgen receptor in this tissue is unknown, and is a topic of continuing research in this laboratory.

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