Specific Progestin Binding in Vitro by Anterior Pituitary and Brain Tissues of Male Rats

YASUO HARUKI, KANJI SEIKI AND YOSHIO IMANISHI

Department of Anatomy, Tokai University School of Medicine, Bohseidai, Isehara City 259-11, Japan

Abstract

Using a highly potent synthetic progestin, R5020 (17α, 21-dimethyl-19-nor-4, 9-pregnadiene-3, 20-dione), the characterization of progestin binding components was attempted in cytosols from the anterior pituitary and various brain tissues of castrated or castrated-adrenalectomized male rats. In both types of operated rats, estrogen administration increased the number of binding sites of R5020 in the anterior pituitary and hypothalamus-preoptic area, but not in the cerebral cortex, amygdala-hippocampus nor in the cerebellum. After incubation of the anterior pituitary cytosol with [3H]-R5020 with or without various unlabeled steroids, a specific progestin binding was found in the 7-8 S region with a binding capacity of 250 fmoles/mg protein and equilibrium dissociation constant of 8 nM. A specific progestin binding was also found in the cytosol from hypothalamus-preoptic area sedimenting in the 7 S region with a binding capacity of 150 fmoles/mg protein and a dissociation constant of 6 nM. Specific binding components in these tissues were heat-labile and protein in nature. Little or no 7 S binding was found in the other brain tissues. Although 4-5 S binding in the cerebral cortex and 4 S binding in the amygdala-hippocampus and cerebellum were found, binding specificity in those tissues was low.

It was concluded that the pituitary gland and hypothalamus-preoptic area of male rats contained progestin receptor in their cytoplasms.

It is generally accepted that the binding of sex steroid hormones by specific receptor molecules represents an initial step in their mechanisms of action on target cells. In spite of many publications on specific uptake and binding of progestin in the peripheral target tissues [see reviews by Liao (1975) and Feder and Marrone (1977)], there is still controversy concerning evidence on the uptake and binding of this hormone by the pituitary gland and brain structures (Seiki and Hattori, 1973; Atger et al., 1974; Kato, 1975). However, Seiki et al. (1977) and Lee et al. (1979), using progesterone, recognized a specific progesterone-binding receptor in the hypothalamus of estrogen-primed female rats. Using a synthetic progestin, R5020 (17α, 21-dimethyl-19-nor-4, 9-pregnadiene-3, 20-dione), Kato and Onouchi (1977), Kato et al. (1978), MacLusky and McEwen (1978), Moguilewsky and Raynaud (1979a, 1979b) and Seiki et al. (1979) reported similar characteristics for a progestin receptor in cytosols from the pituitary gland and hypothalamus of estrogen-primed female rats. The binding specificity of R5020 in the mammalian is restricted to the progesterone receptor but with an association constant of two to five times that of progesterone (Philibert and Raynaud, 1973, 1974).

The presence of progestin receptors in
the pituitary gland and hypothalamus is very important in understanding the mechanism of action of the hormone on the secretion of pituitary gonadotrophins and behavioral responses. The present study was therefore carried out to characterize properties of this hormone receptor in the pituitary gland and brain tissues of castrated-adrenalectomized estrogen-primed male rats.

Materials and Methods

Chemicals

[17α-methyl-3H]17α, 21-dimethyl-19-nor-4, 9-pregnadiene-3, 20-dione ([3]R5020, 87 Ci/mmol) was obtained from New England Nuclear Corporation, Boston. Progesterone, 5α-dihydroprogesterone, estradiol-17β, estradiol benzoate, testosterone and corticosterone were purchased from Sigma Chemical Company, St. Louis, and R5020 from New England Nuclear Corporation.

Animal treatment and cytosol preparation

Male Wistar rats, weighing about 200 g, were castrated (Cx), or Cx and adrenalectomized (Ax) for 13 days prior to use. Before sacrifice, they were sc injected for 3 days with 30 μgiclay of estradiol benzoate in 0.1 ml sesame oil, or the oil vehicle alone. Twenty-four hours after the final injection, the animals were decapitated, and their brains and pituitary glands removed. According to the map of the rat brain (Konig and Klippel, 1974), the brains were dissected into the hypothalamus including preoptic area, cerebral cortex of parietal lobe, amygdala including a part of hippocampus, and cerebellar hemisphere. Anterior lobes of the pituitary glands were then removed. Cytosol fractions from these tissues were prepared in a cold buffer, pH 7.4, consisting of 10 mM Tris-HCl, 1 mM EDTA, 12 mM thioglycerol and 10% (v/v) glycerol as previously described (Seiki et al., 1979), and the cytosol protein concentration was measured by the method of Lowry et al. (1951).

Time course of binding

Cytosols from each tissue of Cx-Ax estrogen-primed animals were incubated for 1 to 9 hr with 500 pm [3H]R5020 with or without an excess of non-radioactive R5020. Pilot studies indicated that 500 pm radioactive R5020 produced saturation binding to each tissue cytosol, and this concentration was selected here for the binding assay. To measure binding, the incubated cytosols were treated with 0.05% dextran-0.5% charcoal, and the resulting supernatant was offered for radioactivity measurement (Seiki et al., 1979).

Effect of estrogen-priming on [3H]R5020 binding to tissue cytosols and blood sera

Cytosols from each tissue and blood sera (4.5 mg protein/ml, diluted with the buffer) of Cx-, or Cx-Ax-estrogen-primed animals were incubated for 3 hr with 500 pm [3H]R5020 with or without an excess of radioinert R5020. The concentration of radioactive R5020 used here was selected for the same reason as given above.

Sucrose density gradient assay

Cytosols from each tissue of Cx-Ax estrogen-primed rats were incubated for 3 hr with 2 nM [3H]R5020 with or without an excess of various non-labeled steroids. A portion (0.3 ml) of each incubate was layered on a linear 5-20% (w/v) sucrose density gradient in the buffer containing 2 nM [3H]R5020 (with or without an excess of unlabeled steroids), and we then proceeded to the density gradient assay (Seiki et al., 1979). Approximate sedimentation coefficients (S) were determined by the method of Martin and Ames (1961), using bovine serum albumin (BSA) and human gamma globulin (HγG) as standards.

Scatchard plot analysis

Cytosols from the hypothalamus-preoptic area and anterior pituitary of Cx-Ax estrogen-primed rats were incubated for 3 hr with 0.1-100 nM [3H]R5020 with or without an excess of unlabeled R5020. Specific binding in each incubate was determined as in Time course of binding, and the results were represented in a Scatchard plot (Scatchard, 1949).

Effect of enzymes and heat

Cytosols from the anterior pituitary and hypothalamus-preoptic area of Cx-Ax estrogen-primed animals were incubated for 3 hr with 2 nM [3H]R5020. A portion (0.3 ml) of the incubates was treated with 50 μg deoxyribonuclease (2,197 U/mg, ribonuclease free), 15 μg ribonuclease (protease and salt free) or 100 μg protease (streptomyces griseus origin) at 18-20°C for 30 min. Another portion (0.3 ml) of the incubates was kept at 60°C for 5 min. Each was then layered on the 5-20% (w/v) sucrose gradient containing 2 nM [3H]R5020, and applied to the ultracentrifugal analysis as described above.

Steroid analysis by thin-layer chromatography

Radioactivity corresponding to the peak at 7 S (fractions 15-22) in the density gradients of [3H]-R5020-incubated cytosols from the anterior pituitary and hypothalamus-preoptic area of Cx-Ax estrogen-primed rats was applied for thin-layer chromatography to analyse steroid composition as previously described (Seiki and Hattori, 1973).
Results

Time course of binding
Specific $[^3H]R5020$ binding to cytosol components in the anterior pituitary and brain tissues reached a plateau by 3 hr at 0–4°C, and the amount of binding remained constant up to 9 hr (Fig. 1). Therefore, for assay purposes an incubation time of 3 hr was chosen.

Effect of estrogen-priming on $[^3H]R5020$ binding to tissue cytosols and blood sera
Approximately the same low levels of $[^3H]R5020$ binding sites in each tissue cytosol and blood serum were observed in both Cx rats and Cx-Ax rats (Fig. 2). The levels of specific binding sites in those samples were in the following order: cerebral cortex > hypothalamus-preoptic area > amygdala-hippocampus > anterior pituitary > cerebellum > serum. In both types of operated rats, however, estrogen administration produced a great increase in the number of binding sites in the anterior pituitary and hypothalamus-preoptic area, and the number of binding sites in those tissues was almost the same in both groups. Estrogen treatment had no effect on the binding sites in the other brain tissues and blood sera at all. The levels of binding sites in those samples changed after estrogen treatment in the following order: anterior pituitary > hypothalamus-preoptic area > cerebral cortex > amygdala-hippocampus > cerebellum > serum.

Sucrose density gradient assay
The sedimentation patterns of cytosols from the anterior pituitary, hypothalamus-preoptic area and cerebral cortex are shown in Fig. 3. There were large peaks of radioactivity in the 7–8 S region in the anterior pituitary and in the 7 S region in the hypothalamus-preoptic area, respectively, both of which were greatly affected by a 100-fold excess of unlabeled R5020, 5α-dihydroprogesterone and progesterone, but not by other steroids used. The 4 S peak in the hypothalamus-preoptic area was not affected by any of the unlabeled steroids. In the anterior pituitary, only a small radioactivity peak was found in the 4 S region. In contrast, the cytosol from the cerebral cortex showed no radioactivity peak in the 7–8 S region, but it showed a radioactivity peak in the 4–5 S region. This peak was slightly affected by the unlabeled progestins and other steroids. A small radioactivity...
Fig. 2. Effect of estrogen-priming on $[^{3}H]$R5020 binding to tissue cytosols and blood sera from Cx or Cx-Ax male rats. Cytosols (4.32 mg protein/ml for each tissue) and blood sera (4.5 mg protein/ml) were incubated for 3 hr with 500 pm $[^{3}H]$R5020 with or without a 100-fold excess of unlabeled R5020. Specific binding was measured using dextran-coated charcoal suspension as described under Methods. Each value is the mean for 2 experiments. (□): Cx, (■): Cx-Ax, (□□): Cx estrogen-primed, (■■): Cx-Ax estrogen-primed. AP: anterior pituitary, HT-POA: hypothalamus-preoptic area, CR: cerebral cortex, AMG-HIP: amygdala-hippocampus, CL: cerebellum.

Fig. 3. Sedimentation patterns of $[^{3}H]$R5020-bound proteins in the cytosols from (a) anterior pituitary, (b) hypothalamus-preoptic area and (c) cerebral cortex of Cx-Ax estrogen-primed male rats. Cytosols (10.7 mg protein/ml for each tissues) were incubated with 2 nm $[^{3}H]$R5020 with or without a 100-fold excess of unlabeled steroids, and were centrifuged in a sucrose density gradient containing 2 nm $[^{3}H]$R5020. The protein concentration in each fraction was monitored by measurement of absorbance at 280 nm (⋯⋯⋯⋯). (●—●): $[^{3}H]$R5020 alone, (○—○): $[^{3}H]$R5020 + R5020, (■—■): $[^{3}H]$R5020 + progesterone, (□—□): $[^{3}H]$R5020 + 5α-dihydroprogesterone, (▲—▲): $[^{3}H]$R5020 + estradiol-17β, (△—△): $[^{3}H]$R5020 + testosterone, (●—●): $[^{3}H]$R5020 + corticosterone. BSA: bovine serum albumin, HγG: human gamma globulin.
peak was found in the cytosols from the amygdala-hippocampus and cerebelum, and this was only slightly affected by progestins (data not shown).

**Scatchard plot analysis**

Figure 4 shows Scatchard plots of $[^{3}H]$-R5020 binding in cytosols from the anterior pituitary and hypothalamus-preoptic area. The plots constructed from the cytosols were rectilinear, and the dissociation constant and number of binding sites of the binders in the anterior pituitary were calculated from the plots to be 8 nM and 250 fmoles/mg protein, respectively. The binding parameters for the cytosol from the hypothalamus-preoptic area were estimated to be 6 nM and 150 fmoles/mg protein, respectively. This indicates the presence of a component with a high affinity and low capacity for R5020 in these tissues.

**Effect of enzymes and heat**

As shown in Fig. 5, $[^{3}H]$R5020 binding to the binding components in cytosols from the anterior pituitary and hypothalamus-preoptic area disappeared after digestion of the cytosols with protease or heating them at 60°C, but did not disappear after treatment with deoxyribonuclease or ribonuclease. This indicates that the binding materials in the tissue cytosols are heat-labile and protein in nature.

**Steroid analysis by thin-layer chromatography**

Thin-layer chromatography of the 7–8 S peak on the density gradients of the cytosol from the anterior pituitary labeled with $[^{3}H]$R5020 clearly revealed that around 90% of the radioactivity was still of unaltered R5020. The same was true for the 7 S peak from the hypothalamus-preoptic area also.
Discussion

The present experiment demonstrates that the anterior pituitary and hypothalamus-preoptic area of male rats contains progestin receptor in their cytoplasms with similar physiochemical binding characteristics to those of progestin receptors in the same tissue cytosols of female rats (Seiki et al., 1977; Kato and Onouchi, 1977; MacLusky and McEwen, 1978; Moguilewsky and Raynaud, 1979a; Lee et al., 1979) and the uterus of the rat, mouse, rabbit and guinea pig (Philibert and Raynaud, 1973; Philibert and Raynaud, 1974). A major difficulty in demonstrating this component was the rapid dissociation of the \([^3\text{H}]\text{R5020}\) receptor complex in male rats. This problem was overcome by using ultracentrifugal assay with sucrose density gradient which already contained radioactive \(\text{R5020}\), as mentioned by Lee et al. (1979).

The very low affinity of estradiol-17\(\beta\), testosterone and corticosterone for the progestin receptors in the anterior pituitary and hypothalamus-preoptic area of male rats indicates that the progestin receptors in these tissues are distinct from the receptors for estrogen, androgen and corticosteroids. These data are consistent with the reports made by Seiki et al. (1977), Kato and Onouchi (1977), MacLusky and McEwen (1978), Seiki et al. (1979) and Moguilewsky and Raynaud (1979a) using the cytosols from the anterior pituitary and/or hypothalamus-preoptic area of estrogen-primed female rats.

It was observed that 5\(\alpha\)-dihydroprogesterone strongly competed with \([^3\text{H}]\text{R5020}\) for binding sites of the progestin receptors in the anterior pituitary and hypothalamus-preoptic area of castrated-adrenalectomized estrogen-primed male rats. The same observations were reported for female rats using \([^3\text{H}]\text{progesterone}\) by Seiki et al. (1977) and Lee et al. (1979), and using \([^3\text{H}]\text{R5020}\) by Kato and Onouchi (1977) and Kato et al. (1978). In adult female rats, Karavolas et al. (1976) and Karavolas et al. (1979) reported that the pituitary and hypothalamic tissues could metabolize progesterone to 5\(\alpha\)-
dihydroprogesterone and these tissues could accumulate 5α-dihydroprogesterone after the injection of either progesterone or 5α-dihydroprogesterone. They suggested from their observations that conversion of progesterone to 5α-dihydroprogesterone might be necessary for the effect of progesterone on these tissues. The present results indicate the possibility that in male rats 5α-dihydroprogesterone acts on the anterior pituitary and hypothalamus-preoptic area through its binding to progestin receptors, as presumed in female rats (Kato and Onouchi, 1977; Kato et al., 1978; Lee et al., 1979).

Approximately the same levels of specific progestin binding sites were measured in the anterior pituitary and brain tissues of castrated male rats as in castrated-adrenalectomized male rats before and after the estrogen-priming (Fig. 2). This may suggest that, not only in female rats (Moguilewsky and Raynaud, 1979b) but also in male rats in the present study, castration and estrogen-priming is sufficient to measure all binding sites including those occupied by progestin secreted by the adrenal gland; adrenalectomy is not necessarily important following castration.

In the castrated-adrenalectomized male rats, estrogen administration increased specific binding of the progestin in the anterior pituitary and hypothalamus-preoptic area, but not in the cerebral cortex, amygdala-hippocampus nor in the cerebellum. This result is consistent with those reported for male castrated rats (Moguilewsky and Raynaud, 1979b) and for female castrated rats (Kato and Onouchi, 1977; MacLusky and McEwen, 1978; Kato et al., 1978; Moguilewsky and Raynaud, 1979a; Moguilewsky and Raynaud, 1979b). The existence of progestin receptors sensitive to estrogen in these tissues of male rats makes one expect that progestins in these tissues facilitate or inhibit estrogen-induced male sexual behavior and gonadotrophin secretion, probably by controlling the concentration of progestin receptors, as reported for female rats (Powers, 1972; Ward et al., 1975; Feder and Marrone, 1977).

The presence of specific progestin receptor in cytoplasms of the anterior pituitary and hypothalamus-preoptic area of castrated-adrenalectomized estrogen-primed male rats supports the views that progestin acts at least at these tissue levels and that some endocrine responses of these tissues to progestin may be mediated by the action of this hormone at the level of the cytoplasm.

References