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SHORT COMMUNICATION

CHARACTERIZATION OF A GLUCOCORTICOID RECEPTOR IN NEONATAL RAT MAMMARY GLAND

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Summary—A glucocorticoid receptor has been identified in cytosolic fractions prepared from 4-day old female Sprague–Dawley rat mammary glands at an early resting stage of mammary development. This component sedimented at 10S and 5S on respectively low and high (0.4 M KCl) ionic strength gradients. It bound dexamethasone with a high affinity ($K_d \sim 2$–6 nM) and a low capacity ($N = 300 \pm 100$ fmol per mg of proteins or 3.3 ± 1.3 fmol per μg DNA), with a hierarchy of affinity by competition studies dexamethasone > corticosterone > progesterone > R 5020 ⋙ Estradiol-17β. The characteristics of this glucocorticoid-binding protein are thus very similar to the adult one isolated from adult rat mammary gland.

INTRODUCTION

Because of their importance in the growth and development of the mammary gland, hormones appear as promoting or permissive agents. It is well known that insulin, growth hormone or prolactin and corticosteroids act in synergy on mammogenesis and stimulate duct growth and branching [1]. Corticosteroids act through specific receptors and the characteristics of mammary glucocorticoid receptors are well documented in adult rats [2, 3] or mice [5] especially during pregnancy and lactation. As far as we know no work has been published on glucocorticoid binding components in the neonatal rat mammary gland. Since many authors studied the glucocorticoid receptor during development of rat liver [6, 7], chick neural retina [8], palatal mesenchymal cells from human embryo [9], we found it interesting to characterize a supposed glucocorticoid-binding component at an early stage when the female rat mammary gland is only composed of a single lactiferous duct that branches into 3–5 secondary ducts.

Our preliminary study demonstrates the existence of a glucocorticoid receptor in 4-day old rat mammary glands, the characteristics of which are very similar to the receptor identified in the functioning gland *viz.* dissociation constant, binding affinities, sedimentation coefficient [2].

EXPERIMENTAL

Animals

Sprague–Dawley rats were obtained from Iffa-Credo (69210-L'Arbresle, France). Animals were killed on day four after birth by cervical dislocation and their mammary glands were excised, frozen on dry ice and stored in liquid nitrogen until receptor assays were performed.

Buffers

Phosphate buffer (5 mM sodium phosphate, 10 mM thioglycerol and 10% glycerol, pH 7.4) and Tris buffer (25 mM Tris–HCl, 1.5 mM EDTA, 10 mM thioglycerol and 10% glycerol, pH 7.4). 10 mM sodium molybdate was added to every homogenizing buffer before use.

Steroids and chemicals

[1,2,4,6,7-^{3}H]Dexamethasone (80 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. [^{14}C]Methylated γ-ovalbumin and globulin were from New England Nuclear Corp. Unlabeled steroids were from Sigma Chemical Co. and all other reagents were from Merck, Darmstadt, Federal Republic of Germany. The Bio-Rad Laboratories protein assay kits were from Touzart et Matignon (Vitry-sur-Seine, France).

Preparation of cytosol and glucocorticoid receptor assays

Mammary glands were homogenized in 3 vol (w/v) of buffer (either Tris buffer for binding studies or phosphate buffer for sucrose gradient analysis) and centrifuged at 105,000 *g* for 1 h to obtain the cytosol—0.5–1 g of frozen powder were necessary to obtain a final cytosolic protein concentration ranging between 3–5 mg/ml. The cytosols were labeled with specified concentrations of [^{3}H]dexamethasone alone or with a 200-fold excess of unlabeled dexamethasone. Specific binding of dexamethasone was estimated by the dextran-coated charcoal (DCC) assay [10] and binding capacity and equilibrium dissociation constant (K_d) were computed by Scatchard analysis of the data [11]. Sucrose gradient analyses were performed according to Eckert and Katzenellenbogen[12]. Aliquots of labeled cytosols were exposed to a DCC pellet to remove free steroid and centrifuged on 5–20% sucrose gradients in phosphate buffer at 280,000 *g* for 20 h at 4°C in a SW 41 Ti Beckman Rotor. The sedimentation coefficients of the glucocorticoid receptor were determined by the method of Martin and Ames[13] and protein concentrations according to the Coomassie blue absorption method [14] with bovine plasma albumin as standard. DNA concentrations were measured as described by Burton[15].

RESULTS

Binding studies

The characteristics of [^{3}H]dexamethasone binding to mammary gland cytosol from 4 experiments with 4-day old

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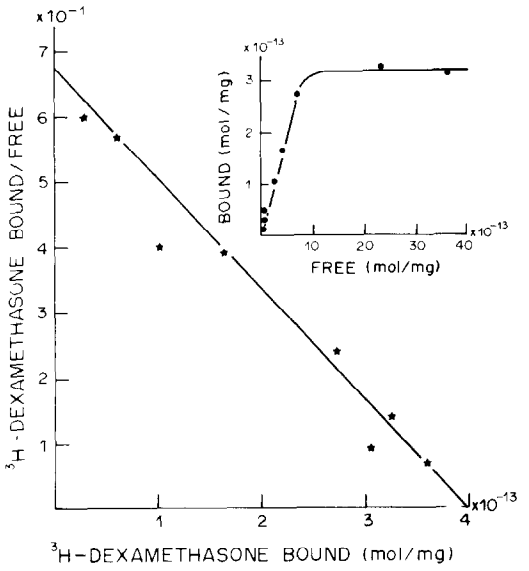

Fig. 1. Scatchard plot of [^{3}H]dexamethasone binding. Cytosol was incubated in duplicate for 18 h at 4°C with increasing concentrations of [^{3}H]dexamethasone (0.5–0.55 nM) with or without a 200-fold excess of unlabeled dexamethasone to determine specific binding. Scatchard analysis (★——★) (lower section) was performed on the specific binding data. In this study K_d = 5.9 nM; sites per mg protein = 400 fmol. A saturation curve of the saturable binding of [^{3}H]dexamethasone against the concentration of unbound dexamethasone is depicted in the inset. (●——●).

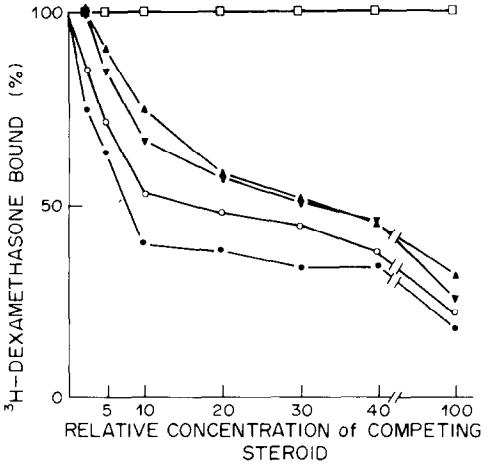

Fig. 2. Comparison of the relative ability of various unlabeled steroids to compete for specific [^{3}H]dexamethasone binding. Aliquots of cytosols from 4-day old rats were incubated 18 h at 4°C with 10 nM [^{3}H]dexamethasone either alone or in the presence of varying concentrations of unlabeled steroids: Dexamethasone (●——●); corticosterone (○——○); progesterone (▼——▼), R 5020 (▲——▲); Estradiol-17β (□——□). Specific binding of [^{3}H]dexamethasone in the absence of competing steroid is taken as 100% and binding in the presence of competing steroids plotted as a percentage thereof. Each point represents the mean value of three separate sets of duplicate determinations.

female Sprague–Dawley rats suggest that specific saturable binding sites of high affinity and low capacity were present (Fig. 1). Inset panel shows effects of increasing concentrations of free [^{3}H]dexamethasone on the amount of the labeled bound hormone. The resulting curve is typical of a saturable event. The lower panel shows the transformation of this data into a Scatchard plot which suggests that a single class of sites is responsible for the specific glucocorticoid binding in this tissue with a $K_d \sim 2$ to 6 nM and capacity of 300 ± 100 fmol per mg protein.

Competition studies

The effect of different steroids on [^{3}H]dexamethasone binding was studied. Figure 2 shows the specificity of

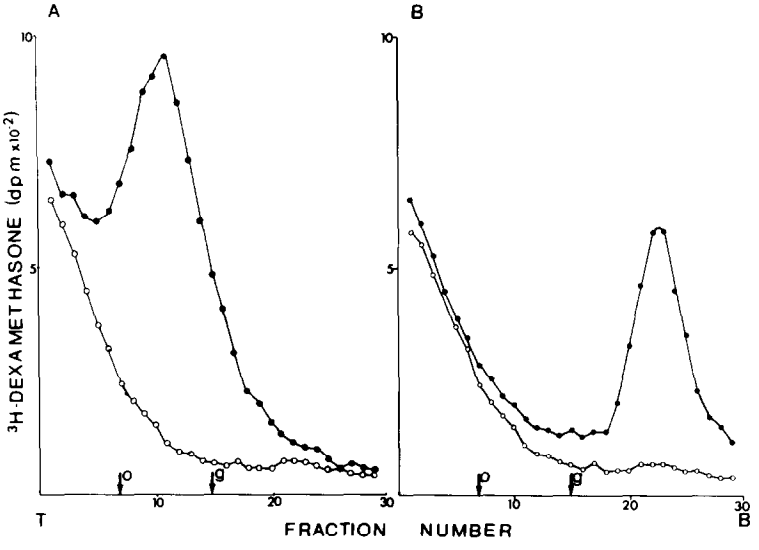

Fig. 3. Sedimentation profiles of mammary glucocorticoid receptors from 4-day old Sprague–Dawley rats. Cytosols were prepared in phosphate buffer containing 10 mM molybdate. The arrows represent the position of standards G – gammaglobulin 7.0 S; O – ovalbumin 3.7 S; B and T refer to the bottom and top of the gradient, respectively. Cytosol was incubated with 10 nM [^{3}H]dexamethasone alone (●——●) or also with a 200-fold excess of unlabeled dexamethasone (○——○) and centrifuged on a linear 5–20% sucrose gradient of low (panel B), or high (0.4 M KCl) ionic strength (panel A).

[^{3}H]dexamethasone binding to mammary tissues from neonatal rats. The hierarchy of competitor effectiveness was dexamethasone > corticosterone > progesterone > R 5020. No binding inhibition was observed with estradiol-17β. Unlabeled dexamethasone was ~1.3 times as potent as corticosterone in competing with [^{3}H]dexamethasone binding sites, ~1.8 times as progesterone and ~2.3 times as R 5020.

Sucrose density gradient analysis

The sucrose gradient analysis of labeled dexamethasone is shown in Fig. 3. A 10S glucocorticoid binding component can be seen in panel B; in high salt ionic strength (Panel A) the binding activity shifts to 5S. These peaks are both completely displaced when excess unlabeled steroid is included in the incubation medium prior to the application on the gradients.

DISCUSSION

Our study shows the existence of a specific glucocorticoid binding protein in mammary gland from 4-day old female rats. Its physicochemical characeristics appear similar to those previously published for adult rat mammary gland. Quirk *et al.*[2] described a single class of low capacity binding sites with a dissociation constant K_d of ~1 nM in cytosols from both pregnant and lactating rat epithelial cell clusters, and noted a doubling of the number of sites per cell in lactating rat mammary glands. These results are in agreement with Haslam *et al.*[5] who claimed that there is no real difference in the intrinsic dissociation constant for dexamethasone binding to the cytoplasmic glucocorticoid receptor from intact, ovariectomized or lactating mouse mammary gland and confirmed that changes in binding capacity occur under different hormonal backgrounds. Kelly *et al.*[10] also noted these variations in pregnant or lactating rabbit mammary gland. Kalimi[16] concluded that no age related changes occurred in *in vivo* and *in vitro* nuclear binding of [^{3}H]dexamethasone receptor complexes in kidney, testis and heart. Aging should not alter glucocorticoid receptor concentration, affinity or nuclear binding ability. However, it seems that liver glucocorticoid receptors of fetal and neonatal rats are physicochemically different from those of adult rats [7] *viz.* the stability of steroid bound or free receptor forms on gel filtration and DEAE-cellulose patterns. But the dissociation constant remains unchanged. Giannopoulos[6] postulated the existence of dissimilar receptors in fetal and adult liver, or the presence of more than one receptor site detectable 1 or 2 h after birth increasing in number between the second and the fifth day. Our results showed the presence of specific saturable binding sites of high affinity at day 4 of postnatal life (with a similar constant of dissociation on the day of birth—not reported here). The number of binding sites found in the present study appears to be higher in the early postnatal rat mammary gland than in the adult one. It is important to point out that the values reported for glucocorticoid receptors do not represent the total cytosolic receptor levels, for at 4°C only 60–75% exchanges between bound and free hormone are known to occur. In addition, only cytosolic receptor variations are reported and these concentrations may reflect changes in the nuclear transfer of receptors under varying concentrations of circulating hormones. It is clear that on day 4 of postnatal life at a time when the mammary gland is still poorly differentiated and non functional, the low plasma level of free corticosterone [17] results in a higher level of free cytosolic glucocorticoid receptors. These receptors are therefore present just before the dramatic rise in plasma corticosteroid level which occurs shortly afterwards [17].

Our competition study appears identical to those realized on adult rat mammary glands. It is noteworthy that the specific progestin R 5020 is less competitive than unlabeled dexamethasone for specific [^{3}H]dexamethasone binding. We have excluded DTT from the homogenizing buffer so that R 5020 only bound to a single class of high affinity sites, characteristic of progesterone receptors [5] and thus prevented any contamination of class I glucocorticoid binding sites by progestins [4], conforming the hypothesis of Haslam[5].

By the addition of molybdate to every homogenizing buffer, we obtained a stabilization of the glucocorticoid receptor which sedimented as expected at 10S on low salt gradients [18]. In contrast to Muldoon's hypothesis [20] no 4S form could be observed, thus confirming the better suitability of phosphate buffer for preserving the integrity of the "native" receptor form [19] which is already present at this early stage of life. The studies presented in this paper identify and characterize a glucocorticoid binding protein in the postnatal rat mammary gland cytosol which seems to be very similar to the classical glucocorticoid receptor demonstrated in adult gland by its binding affinities and by several physicochemical properties (K_d, sedimentation coefficient). Further work is needed to determine if the intrinsic properties of the glucocorticoid receptor in adult and neonatal rat mammary glands are identical and to determine the time of its appearance during fetal life.

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