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OESTROGENS DOWN-REGULATE TYPE I BUT NOT TYPE II ADRENAL CORTICOID RECEPTORS IN RAT ANTERIOR PITUITARY

MÓNICA FERRINI, ANA MARÍA MAGARIÑOS and ALEJANDRO F. DE NICOLA*
Laboratorio de Bioquímica Neuroendócrina. Instituto de Biología y Medicina Experimental, and Fundación Raquel Guedikian de Estudios sobre el Estres, Buenos Aires, Argentina

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Summary—We have studied type I and type II adrenal cortical steroid receptors in the anterior (AL), intermediate (IL) and posterior (PL) lobes of the pituitary and in the hippocampus of ovariectomized-adrenalectomized female rats and in castrated-adrenalectomized male animals, with or without oestrogen treatment. Using [^{3}H]dexamethasone as ligand and conditions suitable for determination of its binding to type I and type II receptors, we found that 4 or 15 days of oestrogen reduced type I receptors in AL by 50–60% without changes in IL, PL or hippocampus, or in type II sites in any of the four neuroendocrine tissues studied. This down-regulatory effect was seen only in female rats and no change was found for males. The reduction in type I sites in AL in oestrogenized female rats was confirmed by labelling type I sites with the synthetic antimineralocorticoid [^{3}H]ZK 91587. Saturation analysis with [^{3}H]ZK 91587 demonstrated that the reduction was due to a reduction in B_{max} without change in K_d. We conclude that: (a) type I receptors in the anterior pituitary are under oestrogenic control; (b) there is a sex difference in the response to oestrogen of AL type I sites; and (c) this demonstration may be useful in determining the role of type I receptors in neuroendocrine regulation of the anterior pituitary by hormones derived from the adrenal cortex, and the participation of sex hormones in this process.

INTRODUCTION

It is accepted that there is a marked sex difference in adrenal cortical function in rats, and several reports have pointed out that gonadal influences on adrenal secretory activity are mediated at several levels. Thus, while some effects of sex hormones can occur directly at the adrenal level or in the periphery [1–3], other actions affect the central mechanisms modulating ACTH secretion [4, 5].

This last aspect, namely, control of ACTH synthesis and secretion, is a complex multifactorial process involving stimulatory and inhibitory substances and is reviewed in Ref. [6]. Among the latter factors, adrenocortical steroids are known to negatively regulate the mechanisms leading to ACTH synthesis and release (reviewed in Refs [6, 7]). In this regard, corticoids have been shown to bind to intracellular receptors located in the anterior pituitary, the hypothalamus and the limbic system [8]. Current hypotheses of the mechanism of hormonal action in neuroendocrine tissues recognize a type I receptor which displays high affinity for natural hormones, and a type II receptor showing low affinity for these hormones but preference for synthetic compounds [9, 11].

Given the reports of sex differences in the control of central nervous system–pituitary–adrenal axis, the effects of sex hormones upon adrenocortical steroid binding activity in brain [12, 13] and oestrogenic effects on mRNA for corticoid receptors in pituitary lobes [14, 15], we have determined the content of type I and type II receptors in the anterior lobe (AL), intermediate lobe (IL) and posterior lobe (PL) and in hippocampus from animals depleted of endogenous oestrogens by gonadectomy, with or without oestrogen replacement. The aim of these experiments was to explain the hormonal control of receptor activity, which in turn may provide insights into the role of receptor subtypes in hormonal effects on the pituitary and the brain.

EXPERIMENTAL

Experimental animals

Female (150–200 g) and male (250–300 g) Sprague–Dawley rats were used. Animals were housed in a constant temperature room (24°C) with a controlled light–dark cycle (lights on 05.00–19.00 h) provided with laboratory rat chow and water *ad libitum*. Rats were ovariectomized (OVX) or castrasted (GDX) under ether anaesthesia either 6 or 17 days before

*To whom correspondence should be addressed: Dr A. F. De Nicola, Laboratorio de Bioquímica Neuroendócrina, Instituto de Biología y Medicina Experimental, Obligado 2490 (1428) Buenos Aires, Argentina.

killing; 48 h before the experiment bilateral adrenalectomy (ADX) was carried out to avoid occupation of glucocorticoid receptors by endogenous hormones. When gonad-intact rats were used, they were ADX only. After ADX, rats were given 0.9% NaCl as drinking fluid.

Oestrogn administration was as follows: in some OVX or GDX rats, oestradiol benzoate (E_2B) dissolved in vegetable oil was given s.c. in doses of 25 μg/kg body wt starting 2 days after OVX or GDX, for 4 days [16]. In another treatment schedule, OVX rats were given 25 μg/kg body wt of the long-acting ester oestradiol valerate (E_2V) s.c. daily for 15 days, starting 2 days after OVX. At the end of this period rats were killed by decapitation and the pituitary and brain removed. The dorsal hippocampus was dissected from the brain in some groups of rats; the AL, IL and PL were separated from the pituitary glands with watchmaker's forceps under a stereomicroscope. To assess the adequacy of separation of the pituitary lobes differential histological staining with haematoxylin-eosin-alcian blue was used according to Herlant[17]. This procedure, performed on microtome sections of tissue blocks embedded in paraffin, showed minimal or no contamination of each pituitary lobe with neighbouring tissue.

Receptor binding assays

AL pooled from 3 rats, IL or PL pooled from 7 rats, and hippocampus from 1 rat were used per assay. Tissues were homogenized in 300 μl of 10 mM Tris–HCl, pH 7.4, 2 mM mercaptoethanol, 1.5 mM EDTA, 10% glycerol, 20 mM sodium molybdate, as previously described [18, 19]. The homogenates were centrifuged [20] to obtain a soluble cytoplasmic extract. 70 μl aliquots of this extract containing average levels of protein of 6.5 mg/ml for AL, 3 mg/ml for IL or PL, and 5 mg/ml for hippocampus were incubated with tritiated ligands to determine levels of type I and type II adrenal corticoid receptors. In the first series of studies, incubations contained 20 nM [^{3}H]dexamethasone (DEX) alone (i), plus 250-fold excess of the antiglucocorticoid RU 28362 (ii), or plus 250-fold molar excess of both RU 28362 and corticosterone (B) (iii). Thus (i) minus (ii) would yield binding to type II receptors, whereas (ii) minus (iii) binding to type I receptors, as previously described [19]. In a typical experiment, total bound cpm in AL ranged from 2500 to 3200 cpm, whereas radioactivity resistant to addition of RU 28362 and B, i.e. non-specific binding, represented less than 13% of total bound [^{3}H]DEX.

In the second series of experiments, cytoplasmic extracts were incubated with the antimineralocorticoid [^{3}H]ZK 91587, a high affinity ligand for type I sites [21] and Grillo *et al.*, unpublished observations). For single point assays, incubations contained 5 nM [^{3}H]ZK 91587 in the presence or absence of 1000-fold molar excess aldosterone to measure non-specific binding, which was subtracted from total to give specific binding. For saturation analysis, 0.1–10 nM [^{3}H]ZK 91587 were incubated, and binding parameters (K_d and B_{max}) were determined by the method of Cressie and Keightley[22].

All incubations were at 0–4°C for 20 h, at the end of which bound and free steroid were separated on Sephadex LH-20 minicolumns [18]. Column eluates were collected and radioactivity was measured by liquid scintillation spectrometry. Results were converted into fmol [^{3}H]steroid bound per mg protein; proteins and DNA were determined by the methods of Lowry *et al.*[23] and Burton[24], respectively.

Materials

[6,7-^{3}H]DEX (SA 46 Ci/mmol) was purchased from Amersham (Buckinghamshire, England); [^{3}H]-ZK 91587 (7α-methoxy-carbonyl-15, 16-methylene-3-oxo-17α pregn-4-ene-21-17-carbolactone, SA 70 Ci/mmol) was obtained from New England Nuclear (Boston, Mass, U.S.A.). RU 28362 was the kind gift of Dr D. Philibert, Roussel-Uclaf (France) and E_2B and E_2V were gifts from Gador Laboratories and Schering Argentina, respectively.

RESULTS

In experiments performed in ADX rats of both sexes, whether the animals were gonadectomized or intact, adrenal corticoid receptors (type I as well as type II sites) were 2–3-fold higher in AL than in IL or PL. Reports in the literature have shown preponderance of glucocorticoid receptor levels in AL over the other pituitary lobes [25–27].

Figure 1 shows results of type I and type II receptor determination using [^{3}H]DEX as ligand, for OVX-ADX rats receiving E_2B for 4 days and their respective controls. Oestrogen administration did not affect either receptor type in IL, PL or hippocampus; in AL, however, E_2B produced a $\sim$50% lowering of type I receptors ($P < 0.05$). When oestrogenization was extended to 15 days with the long-acting oestrogen E_2V (Fig. 2), similar results were obtained, i.e. an even more pronounced reduction in type I receptors was found in AL (66%, $P < 0.001$) with no changes in type I sites in IL, PL or hippocampus, or in type II sites in the four tissues studied. However, as oestrogen increases AL weight and protein content, reductions in type I receptors could reflect constant receptor levels but increased protein levels, without real changes per target tissue cell. To exclude this possibility, we have recalculated the data per μg DNA and per pituitary lobe. Results in Table 1 show that the fall in type I receptors persisted, when results were expressed per mg protein, per μg DNA or per AL lobe.

To ascertain whether type I sites were down-regulated by oestrogens given only at pharmacological doses [16] or whether similar results could be obtained in a more physiological model, the effect of OVX was assessed. Figure 3 shows that gonad-intact

female rats had lower levels of type I receptors in AL ($P < 0.05$) but not in IL or PL, compared with OVX rats. Confirming the results shown in Figs 1 and 2, type II receptor levels—although highly variable from animal to animal—did not differ between the two groups. The variability in type II receptors in hippocampus from females was noteworthy (Figs 1 and 2). It is possible that time after OVX had some influence, considering that animals used for the experiments of Fig. 1 were OVX for 4 days, whereas 15 days elapsed for those of Fig. 2. This possibility is being further evaluated in our laboratory.

Since female rats were used above, an investigation was carried out in males, to investigate the possibility of a sex difference in the response to oestrogen. As shown in Fig. 4, the down-regulatory action of oestrogens on type I receptors did not occur in GDX-ADX rats receiving 25 μg/kg body wt E_2B for 4 days, i.e. a treatment that reduced by ~50% type I sites in OVX-ADX females (Fig. 1). As already seen for female rats, however, type I sites in IL, PL and hippocampus, as well as type II site in the four tissues examined, were not changed by oestrogen administration to male rats.

Results shown in Fig. 5 depict the effects of oestrogens given to female rats on type I receptors labeled with the antimineralocorticoid [^{3}H]ZK 91587, a high affinity ligand for type I sites [21]. For these experiments, specific binding of [^{3}H]ZK 91587 was determined in AL, IL, PL, and hippocampus of OVX-ADX rats with or without treatment with E_2B for 4 days. As for the results reported with [^{3}H]DEX under conditions suitable for determination of ligand binding to type I sites (Fig. 1), a significant reduction in type I sites due to E_2B treatment was confined to the AL, without changes in IL, PL and hippocampus. To examine whether this reduction was due to a change in the affinity of the receptor or in the number of receptor molecules, a range of concentrations of [^{3}H]ZK 91587 (0.1–10 nM) was incubated with

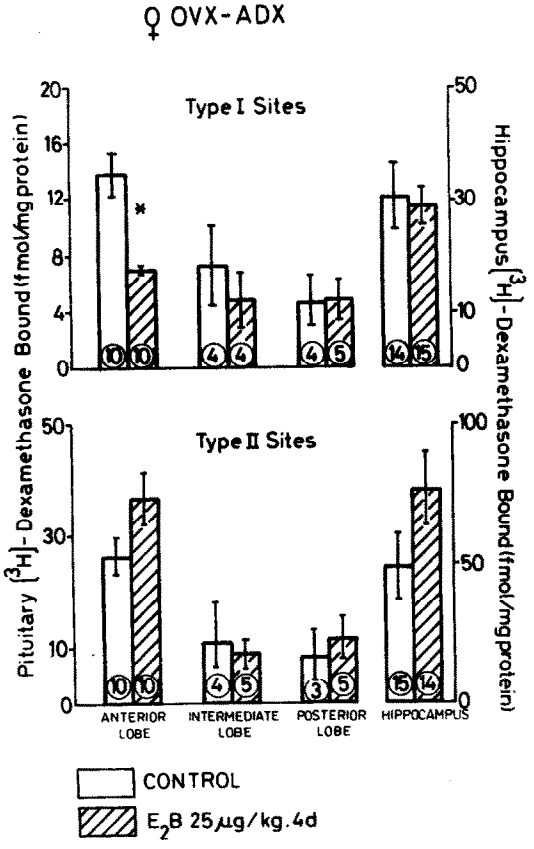

Fig. 1. Effects of 4-day oestrogen treatment of female rats on type I and type II adrenal corticoid receptor binding in anterior, intermediate and posterior lobes of the pituitary and in hippocampus of ovariectomized-adrenalectomized rats (OVX-ADX, control open columns) and in rats given E_2B (25 μg/kg body wt) for 4 days (hatched columns). Binding of [^{3}H]dexamethasone (DEX) to type I sites (upper graph) and type II sites (lower graph) was determined in cytoplasmic extracts of the four neuroendocrine tissues. In these and the following figures, results represent the mean ± SE of the number of determinations shown at the bottom of the columns; each determination was performed with a pool of 3 anterior lobes, 7 intermediate or posterior lobes, and hippocampus from single animals. *Significantly lower than controls ($P < 0.05$).

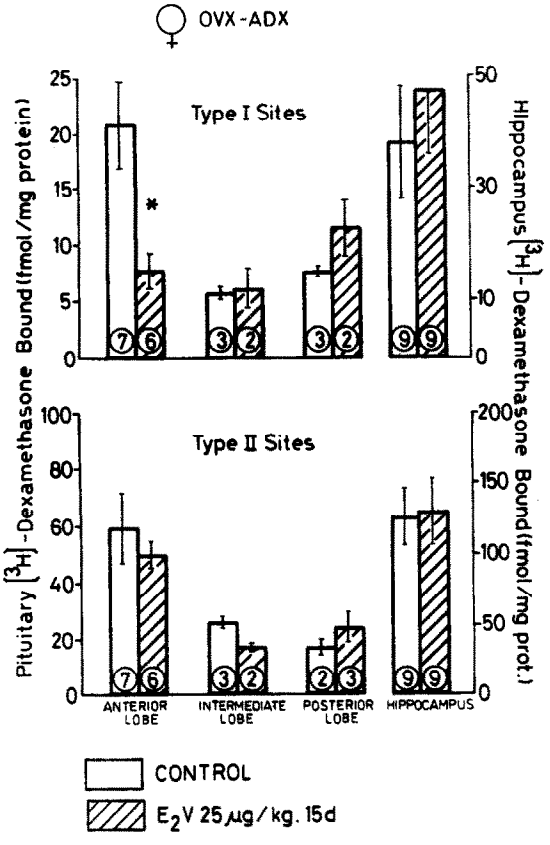

Fig. 2. Effects of 15-day oestrogen treatment of female rats on type I and type II adrenal corticoid receptor binding in anterior, intermediate and posterior lobes of the pituitary and in hippocampus. OVX-ADX rats were used as controls (open columns) or given E_2V (25 μg/kg body wt) for 15 days (hatched columns). Binding of [^{3}H]DEX to type I sites (upper graph) and type II sites (lower graph) was determined. *Significantly lower than controls ($P < 0.001$).

Table 1. Levels of type I adrenal corticoid receptors in anterior pituitary lobes of ovariectomized-adrenalectomized controls (OVX-ADX) and OVX-ADX rats treated with oestrogen

Experiment	Group	[^{3}]Dexamethasone bound to type I receptors fmol/mg prot	fmol/μg DNA	fmol/lobe
I	OVX-ADX	13.90 ± 1.48	0.133 ± 0.013	9.43 ± 0.51
	OVX-ADX + E_2B	7.14 ± 0.70[a]	0.076 ± 0.001[c]	5.28 ± 0.35[d]
II	OVX-ADX	20.80 ± 4.00	0.367 ± 0.07	10.23 ± 1.76
	OVX-ADX + E_2V	7.85 ± 1.74[d]	0.109 ± 0.02[a]	5.92 ± 0.70[b]

Results are the means ± SE of [^{3}H]DEX binding to type I receptors in untreated OVX-ADX rats, and rats treated with 25 μg/kg body wt/day of oestradiol benzoate (E_2B) for 4 days (Experiment I) or the same dose of oestradiol valerate (E_2V) for 15 days (Experiment II). For each assay, anterior pituitary lobes from 3 rats were pooled. [a]$P < 0.05$; [b]$P < 0.02$; [c]$P < 0.01$; [d]$P < 0.001$, vs OVX-ADX rats.

cytoplasmic receptors from AL, dissected from control or oestrogenized ADX-OVX female rats. Figure 6 demonstrates that [^{3}H]ZK 91587 bound to a single receptor class with comparable affinity in both groups (dissociation constant, K_d ~0.9 nM). Number of sites, however, was reduced by 33% in animals receiving oestrogen treatment.

DISCUSSION

Heterogeneity of receptors in neuroendocrine tissue has received considerable attention leading to the recognition of high affinity receptors for natural hormones (B and aldosterone) called type I receptors, and high affinity receptor for DEX called type II receptors [7, 11]. Whereas type I sites are localized mostly in hippocampus, septum and amygdala type II sites are present in high quantities in limbic system, several brain nuclei and AL, which can be regarded as a typical type II receptor containing

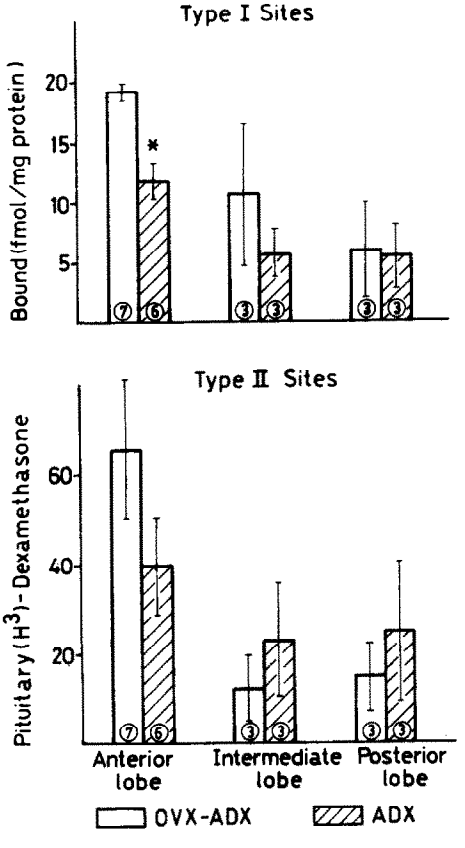

Fig. 3. Effects of OVX of female rats on type I and type II adrenal corticoid receptors in anterior, intermediate and posterior lobes of the pituitary. Rats were OVX-ADX (open columns) or ADX only (hatched columns). Binding of [^{3}H]DEX was determined to type I sites (upper graph) and to type II sites (lower graph). *Significantly lower than controls ($P < 0.001$).

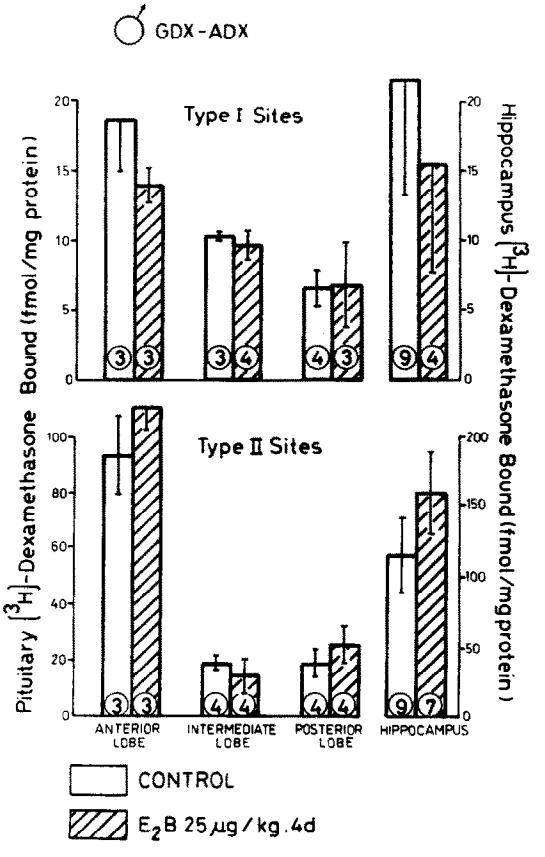

Fig. 4. Effects of 4-day oestrogen treatment of male rats on type I and type II adrenal corticoid receptor binding in anterior, intermediate and posterior lobes of the pituitary, and in hippocampus. Male rats were castrated and adrenalectomized (GDX-ADX, controls open columns) or given E_2B (25 μg/kg body wt) for 4 days (hatched columns). Binding of [^{3}H]DEX was determined to type I sites (upper graph) and to type II sites (lower graph). There were no statistically significant differences between control and oestrogenized males in any of the four neuroendocrine tissues studied.

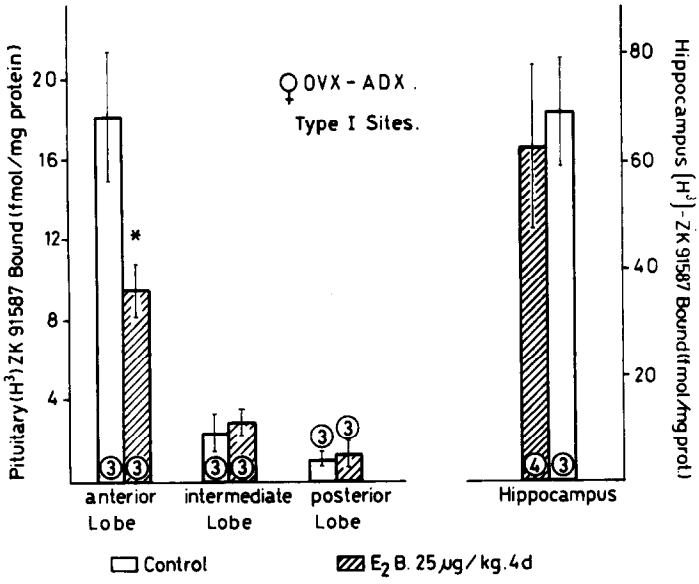

Fig. 5. Effects of 4-day oestrogen treatment of female rats on type I receptors in anterior, intermediate and posterior lobes of the pituitary and in hippocampus. OVX-ADX rats were used as controls (open columns) or given E_2B (25 µg/kg body wt) for 4 days (hatched columns). Type I sites were determined by incubation of cytoplasmic extracts with the antimineralocorticoid [^{3}H]ZK 91587 (5 nM). *Significantly lower than controls ($P < 0.05$).

tissue [8, 18, 28]. In contrast, cells of the IL are devoid of glucocorticoid type II receptors on immunocytochemistry [26], unless the tissue is removed from hypothalamic influences, in which case receptor activity appears [27]. It is possible that our findings of low, but still measurable glucocorticoid receptors in normal IL are due to different limits of detectability provided by biochemical and immunocytochemical techniques. Alternatively, use of whole IL lobes for receptor assays may include not only IL cells but also gonadotrophs, glial cells and pituicytes, which are found scattered in the parenchyma of the IL [29, 30]. In contrast, our results with the pars nervosa are in complete agreement with other reports, as cells of PL

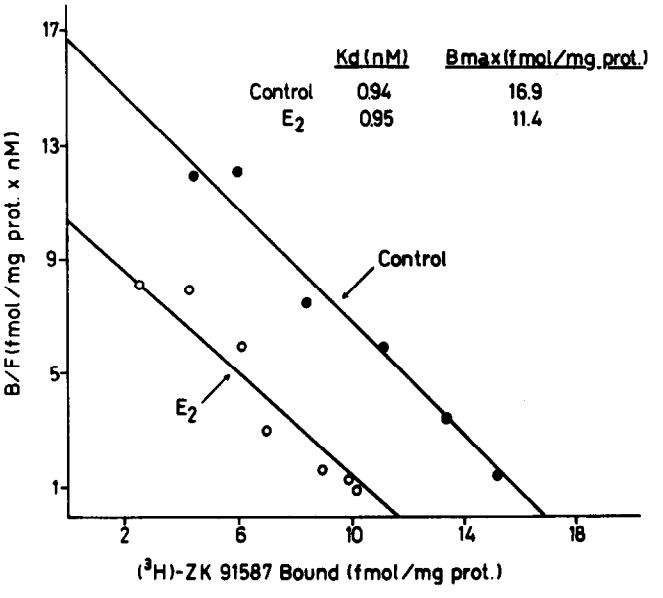

Fig. 6. Scatchard plot analysis of specifically bound [^{3}H]ZK 91587 to type I receptors in anterior pituitary lobe cytoplasmic extracts from OVX-ADX rats (control) and OVX-ADX rats receiving 25 µg/kg body wt E_2B during 4 days (E_2). Cytoplasmic extracts were incubated with 0.1–10 nM [^{3}H]ZK 91587, with parallel incubations containing a 1000-fold molar excess of non-radioactive aldosterone. Binding parameters were calculated by the method of Cressie and Keightley [22]; values for K_d and B_{max} are shown in the graph.

can accumulate systemically injected [^{3}H]DEX [25] and cells identified as pituicytes are immunologically positive for type II receptors [26].

Our results that oestrogen down-regulate type I sites in female AL agree with previous observations of Turner and Ansari[13], in the sense that they found female rats to contain about 67% of the number of high affinity type I receptors compared to male. Our current model suggests that the lower number may be due to oestrogen action in females. However, our data in male AL shows a trend which may become significant with longer treatment, and sex difference may therefore be a matter of sensitivity to oestrogen.

In contrast to Turner and Ansari[13], type II receptors were unmodified by oestrogens, while they reported a slight (20%) reduction in females compared to males. Our work is in agreement with that of Koch *et al.*[31], who found that type II glucocorticoid receptors were similar in the AL of both sexes, although a transcortin-like compound contained in the pituitary was higher in females. Since the ligands used by us—[^{3}H]DEX and [^{3}H]ZK 91587—do not bind to transcortin, the presence of this pituitary binder would not interfere with the reported conclusions.

Sex differences in adrenal steroid receptor binding have already been described by Turner and Weaver[12] in hippocampus, which showed higher [^{3}H]DEX and [^{3}H]B binding in females than in males, whereas the opposite result was obtained for hypothalamus. These findings, however, are difficult to compare with ours in the sense that we did not assay male and female tissues in parallel; in the case of hippocampus, however, we did not observe changes due to oestrogens in either sex. However, Turner and Weaver[12] reported that OVX increased [^{3}H]B binding in female rat hypothalamus. Given that [^{3}H]B is a good ligand for type I receptors [7, 19], the results would imply that oestrogens produced by normal cycling rats affect type I receptor levels, similar to our results in AL.

Pfeiffer and Barden[14, 15] reported that OVX increases mRNA for glucocorticoid type II receptors in AL and IL, whereas oestradiol reversed this action. This conclusion is at variance with our binding assays in the sense that OVX and oestradiol did not modulate type II receptors in AL or IL. The apparent discrepancy may be resolved in that receptor protein assays are not superimposable on measurement of mRNA content. In this context Yang *et al.*[32] have shown that glucocorticoid mRNA distribution in hippocampal subfields only partially corresponds to that measured for the receptor protein, suggesting differences in translational activity or the stability of the gene product.

The biological significance of oestrogenic effects on type I, mineralocorticoid-like receptors is hampered by uncertainty regarding the type of cells in the AL expressing these receptors. Early work by Ermisch and Ruhle[33] reported that a large number of glandular cells of the AL retained [^{3}H]aldosterone, and that preferential concentration of label by any specific cell type has been technically difficult to assess [33]. Our former demonstration of preferential uptake of circulating [^{3}H]aldosterone in AL did not localize the cells concentrating this hormone [34]. Furthermore, mineralocorticoid-receptor-like aldosterone binding proteins were shown in pituitary tumor cells (GC, CH_3) that secrete growth hormone and prolactin but not in ACTH-producing AtT-20 cells [35]. In immature rats, aldosterone affected corticotrophic activity by interacting with glucocorticoid binding sites and not with its specific receptors [36], indicating that corticotrophs may not contain type I receptors. Finally, Krozowski and Funder[37] suggested that a role for type I receptors in AL would be the regulation of a pituitary aldosterone-stimulating hormone, the localization of which is unclear.

On the other hand, type II glucocorticoid receptors are located, in addition to corticotrophs which comprised only 5% of the total cell population, in growth hormone and prolactin cells, since adrenal steroids act on these cells [26, 37, 39, 40]. Immunocytochemically, the number of AL cells staining with an anti-type II receptor antibody also exceeded the number of corticotrophs [26], suggesting different functions for glucocorticoids on pituitary hormone secretion, due to the presence of type II receptors in all cells. However, speculation regarding the role of type I receptors on pituitary function under conditions of high or low oestrogen must await precise cell localization studies, and should be correlated to pituitary hormone secretion assays under the combined action of oestrogen and suitable ligands for the type I receptor.

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