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THE SPECIFICITY OF OESTROGEN RECEPTOR IN BRAIN, PITUITARY AND UTERUS

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1 The specificity of oestrogen receptor in rat brain regions (hypothalamus, amygdala and cortex), pituitary and uterus was studied by measurement of the inhibition of 17β-oestradiol high affinity binding in cytosol, in the presence of unlabelled putative inhibitors of binding.

2 Binding was inhibited only by those of the compounds tested that possessed oestrogen agonist or antagonist activity. The affinities were estimated and the ranking order of the compounds was the same in all tissue sources of cytosol and corresponded to the ranking order of agonist or antagonist potency.

3 There were some significant differences between some of the estimated affinities in different tissues, these being seen most commonly between pituitary and hypothalamus on one hand and uterus on the other.

4 The possibility of heterogeneity of oestrogen receptor is discussed.

Introduction

It is believed that many significant actions of steroid hormones are mediated by the formation of complexes with receptors in cytoplasm; the complexes so formed move to the nucleus where they bind to chromatin; this leads to a modification of genetic expression and thence to the physiological responses which are characteristic of the hormone on that particular tissue. As well as influencing secondary sexual organs, oestrogens affect brain and pituitary, to modify sex-linked behaviour and to control gonadotrophin secretion and cytosols from hypothalamus and pituitary have been shown to contain high affinity receptors for oestrogens (Eisenfeld, 1970; Kahwanago, Heinrichs & Herrman, 1970; Notides, 1970; Vertes & King, 1971; Ginsburg, MacLusky, Morris & Thomas, 1971; 1972).

The specificity of those oestrogenic responses is therefore dependent on that of the initial hormone-receptor interaction. The specificity of the uterine receptor has been studied (Korenman, 1968; 1969; 1970; Notides, 1970; De Sombre, Chabaud, Puca & Jensen, 1971), but little is known about that of the reaction in the central nervous system and pituitary. We describe here the binding affinities of a number of oestrogens, antioestrogens and non-oestrogenic steroids for the cytosol receptors of cerebral cortex, amygdala, hypothalamus and pituitary of male and female rats and for uterine cytosol receptors. As it has been reported (Shani, Givant, Sulman, Eylath & Eckstein, 1971) that perphenazine inhibits the uptake of oestradiol into the hypothalamus we included it in our list of test substances.

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Methods

[2,4,6,7^{3}H]-oestradiol (85 Ci/mmol), obtained from the Radiochemical Centre, Amersham, England, was repurified and stored as described before (Ginsburg, Greenstein, MacLusky, Morris & Thomas, 1974).

Sources of unlabelled steroids and other putative inhibitors are shown in Table 1. They were stored in ethanol and diluted in buffer to the required concentration just before use.

Preparation of cytosol

Phosphate buffer 0.01 M pH 7.4 containing 250 mM sucrose was used. Homogenization medium contained, in addition, 0.1 M 2-mercaptoethanol. Twelve week old female (selected at the metoestrous stage of the 4 day oestrous cycle) and male Wistar rats were beheaded and tissues (cerebral cortex, amygdala and hypothalamus, whole pituitary and uterus) were dissected into ice-cold homogenization medium and homogenized as described elsewhere (Ginsburg *et al.*, 1974). Homogenates were centrifuged (110,000 $r_{av} \times g \times 1$ h) to prepare cytosols.

Measurement of binding

[^{3}H]-oestradiol: equilibrium dissociation constants of reaction were determined as described in detail elsewhere (Ginsburg *et al.*, 1974). In summary, 0.2 ml aliquots of cytosols were incubated (30°C) with 0.1 ml aliquots of [^{3}H]-oestradiol (10^{-9} M to 3×10^{-10} M) in buffer for 10 min (hypothalamus), 20 min (pituitary) and 30 min (cortex and amygdala); times that are more than enough to allow equilibrium between the high affinity receptor and the hormone to be reached. On completion of incubation 0.2 ml aliquots of the incubation were applied to small columns of Sephadex LH20 (6×0.45 cm) and allowed to remain in contact with the gel at 0–4°C for 30–60 min, in which time much of the low affinity bound oestradiol is freed and fixed to the gel although the hormone-receptor complex remains intact. Some radioactivity from the low affinity high capacity complexes appears in the eluates; estimates of eluted oestradiol arising from this source were obtained from parallel incubation in the presence of excess diethylstilboestrol (DES). It is necessary to subtract these from total binding to obtain values for high affinity binding.

Measurement of the binding of unlabelled compounds in the high affinity oestradiol binding system

Affinities were measured by competition with radioactive oestradiol. As well as incubating cytosols with [^{3}H]-oestradiol in the presence and in the absence of an excess of DES, we carried out parallel incubations in the presence of other competitors. Bound radioactivity was then separated from free in the usual way.

Calculation of affinities

[^{3}H]-oestradiol: equilibrium dissociation constants of reaction (K_d), were calculated by the method of Scatchard (1949) who derived the equation $B/F = K_a(n - B)$ to describe the binding of a ligand in a solution of a macromolecule where n is the concentration of equivalent and independent binding sites,

Table 1 Unlabelled steroids and other putative inhibitors used

Trivial name (abbreviation)	*Chemical name*	*Source*
Steroids		
[3]Progesterone	Δ 4 Pregnen-3, 20 dione	Sigma
[3]Corticosterone	Δ 4 Pregnen-11β, 21 diol-3, 20 dione	Sigma
[3]Testosterone	Δ 4 Androsten-17βol, 3 one	Sigma
[3,4]5α-Dihydrotestosterone	5α Androstan-17βol, 3 one	Sigma
[5]Cyproterone	1,2α Methylene-6 chloro-4,6, pregnadien-17αol-3, 20 dione	*Schering Corp.
[4]Androstan-3α diol (3α diol)	5α Androstan-3α, 17β diol	Steraloids
[4]Androstan-3β diol (3β diol)	5α Androstan-3β, 17β diol	Steraloids
[1,4]17β-Oestradiol (17βE_2)	1,3,5 (10) Oestratrien-3, 17β diol	Sigma
[1]17β-Oestradiol (17αE_2)	1,3,5 (10) Oestratrien-3, 17α diol	Sigma
[1]Oestrone (E_1)	1,3,5 (10) Oestratrien-3ol, 17one	Sigma
[1]Oestriol (E_3)	1,3,5 (10) Oestratrien-3, 16α, 17β triol	Steraloids
[1]16-Epioestriol (Epi E_3)	1,3,5 (10) Oestratrien-3, 16β, 17β triol	Steraloids
[2]Ru 2858	11β Methoxy, 17α ethynyl-1,3,5 (10) oestratrien-3, 17β diol	*Roussel
Non-steroidal compounds		
[1]Diethylstilboestrol (DES)	3,4 Bis (4 hydroxyphenyl) Δ 3 hexane	Sigma
[2]MER-25	1 (*p*-2 Diethylaminophenyl)-1 phenyl-2*p* methoxy phenyl ethanol	*Merrell
[2]*cis*-Clomiphene (Cis Clo)	*cis*-Triethyl amino-2(*p*(2-chloro-1, 2 diphenylvinyl)phenoxy) monocitrate	*Merrell
[2]*trans*-Clomiphene (T Clo)	*trans*-Triethyl amino-2(*p*(2-chloro-1,2 diphenylvinyl)phenoxy) monocitrate	*Merrell
Perphenazine	2-Chloro-10 (3-(1-(2 hydroxyethyl)-4 piperazinyl) propyl) phenothiazine	*Schering

* Compounds received as gifts either directly or through the late Dr P.G. MacDonald.
[1] = oestrogens; [2] = antioestrogens; [3] = non-oestrogenic; [4] = testosterone metabolites; [5] = anti-androgens.
The distinction between oestrogens and antioestrogens, on the one hand, and 'non-oestrogens' on the other is of course not absolute. It is made here solely on the basis of primary biological effect. All of the 'non-oestrogens' used will, under certain circumstances, elicit oestrogen or anti-oestrogen-like responses.

where B is the concentration of ligand/macromolecule complex and F is the concentration of free ligand. $K_a = (1/K_d)$ is the equilibrium association constant of reaction. When the ratio B/F is plotted against B the points are fitted by a straight line of slope $-K_a$ and which intercepts the ordinate at nK_a and the abscissa at n.

The affinities of the various competitors were calculated in two ways. Data from preliminary experiments to see whether the effects observed on oestradiol binding were competitive were analysed as follows:

The Edsall & Wyman (1958) equation states that for competitive interaction between a ligand and an inhibitor at a single class of binding sites

$$B = \frac{nK_aF}{1 + K_aF + K_iI}$$

where I = the concentration of free inhibitor and K_i is the equilibrium association constant of the receptor-inhibitor reaction.

By rearrangement, an expression similar in form to the Scatchard equation may be obtained:

$$\frac{B}{F} = \frac{K_a(n - B)}{1 + K_iI}$$

Comparing this with the Scatchard equation we may conclude that the sole effect of a fixed concentration of competitive binding inhibitor on a Scatchard plot of the binding of a ligand to a single class of non-interacting sites, will be to decrease the slope of the plot by a factor of $1/(1 + K_iI)$; neither the rectilinear nature of the plot, nor its intercept where $B/F = 0$, at the saturation binding capacity of the system, will be affected. In contrast, if binding inhibition is due, even in part, to non-competitive interaction then the term $K_a/(1 + K_iI)$ will vary with ligand concentration and the Scatchard plot, in the presence of the inhibitor, will be curvilinear.

At least one experiment was carried out with each inhibitor in which Scatchard plots were constructed from six values of B and F in both the presence and absence of fixed concentrations of inhibitors. For all the compounds that inhibited oestradiol binding, the Scatchard plot was shifted by a linear reflection about the intercept on the abscissa according to the prediction above for competitive inhibition. However, several replications of this procedure for each compound would have been prohibitive in terms of labour and material and our routine practice was to construct the Scatchard plot from 6 values of B and F in the absence of inhibitor and two values for B and F in the presence of a fixed concentration of each inhibitor.

K_{di} (= $1/K_i$) was calculated from the following equation derived from the Edsall & Wyman equation:

$$K_{di} = \frac{1}{nK_a\frac{F}{B} - (K_aF + 1)}$$

thus K_{di} can be obtained if K_a and n are known together with values for F and B in the presence of I.

Results

In preliminary experiments, aliquots of cortex, amygdaloid region, hypothalamus, pituitary and uterus cytosols, from tissues of intact adult female rats were incubated with [^{3}H]-17β-oestradiol (final incubation concentration, 1×10^{-9} M) either alone, or in the presence of a range of concentrations (10^{-10} to 10^{-6}M) of each of the unlabelled test compounds. Control incubates, in each case, contained the same concentrations of [^{3}H]-17β-oestradiol alone and with the various unlabelled compounds, in the presence of excess (10^{-7} M) unlabelled DES. Following incubation, bound [^{3}H]-17β-oestradiol was separated from free steroid by Sephadex LH20 gel filtration, and high affinity binding assessed as previously described.

Results for each tissue cytosol studied were essentially similar: in all cases low affinity binding i.e. binding remaining in the presence of excess DES, was unaffected by the presence of any of the unlabelled compounds, even at the highest concentrations used. In contrast high affinity binding was inhibited, increasingly as the concentration was raised, of unlabelled 17β-oestradiol, diethylstilboestrol, 17α-oestradiol, oestrone, oestriol, 16-epioestriol, Ru 2858, MER-25, *cis*-clomiphene, *trans*-clomiphene and androstan-3α diol. The remaining unlabelled compounds (corticosterone, progesterone, testosterone, 5α-dihydrotestosterone, cyproterone and perphenazine) did not significantly inhibit high affinity binding of [^{3}H]-17β-oestradiol, in any cytosol fraction, at concentrations up to 10^{-6} M.

The nature of the interactions between [^{3}H]-17β-oestradiol, and the various unlabelled compounds found to inhibit high affinity cytosol binding was then studied further, as follows: from the results of the preliminary series of experiments, concentrations of each of the unlabelled inhibitors were chosen (Table 2) which produced only a partial inhibition of the high affinity binding of [^{3}H]-17β-oestradiol in cytosol incubate at a concentration 1×10^{-9} M. Isotherms were then constructed for binding of [^{3}H]-17β-oestradiol to the high affinity sites in each of the tissue cytosols, in the presence and absence of these concentrations of the inhibitors.

Figure 1 shows Scatchard representations of the isotherms obtained with hypothalamic cytosol, in the presence and absence of unlabelled androstan-3α,17β diol (3.3×10^{-7} M). Both sets of data yield rectilinear Scatchard plots, intercepting the abscissa at the saturation binding capacity of the high affinity sites; however, in the presence of the inhibitor, the slope of the plot is markedly reduced. These results are consistent with a model for competitive interaction

Table 2 Concentrations of unlabelled compounds found in preliminary experiments to inhibit high affinity binding of [^{3}H]-17β-oestradiol (5×10^{-10} M) in uterine cytosols, by between 30 and 70%

	(M)
MER-25	3.3×10^{-7}
Androstan-3α diol	3.3×10^{-7}
trans-Clomiphene	3.3×10^{-7}
Androstan-3β diol	3.3×10^{-7}
cis-Clomiphene	3.3×10^{-8}
17α-Oestradiol	3.3×10^{-8}
Oestrone	3.3×10^{-8}
Oestriol	3.3×10^{-8}
16 Epioestriol	3.3×10^{-9}
Ru 2858	3.3×10^{-9}
17β-Oestradiol	1.3×10^{-9}
Diethylstilboestrol	6.7×10^{-10}

between androstan-3α,17β diol and [^{3}H]-17β-oestradiol for the available binding sites.

Similar results were obtained with all other combinations found to inhibit [^{3}H]-17β-oestradiol binding with cytosols: from the 9 different classes of tissue source and with each of the 12 compounds, Scatchard representations of the binding isotherms were in all cases consistent with binding inhibition being due to competitive interaction between the inhibitors and [^{3}H]-17β-oestradiol at the high affinity sites. These experiments yielded values for K_{di} and for each compound up to 4 additional estimates of the parameter were obtained by the more economical double point method.

The results were satisfactorily concordant with all tissues except cortex where the variability of the double point assays was unacceptably high. In view of the extremely low levels of high affinity binding

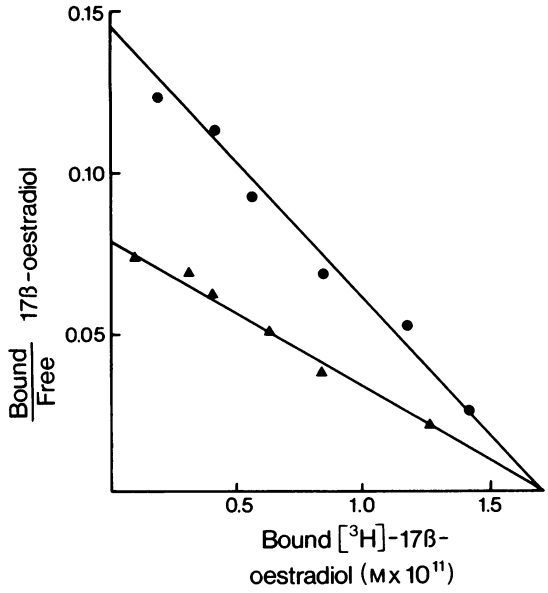

Figure 1 Inhibition of [^{3}H]-17β-oestradiol binding in female rat hypothalamic cytosol by androstan-3α diol (3.3×10^{-7} M): (●) [^{3}H]-17β-oestradiol alone; (▲) [^{3}H]-17β-oestradiol plus androstan-3α diol.

present in cortex cytosols, this problem was ascribed to errors in the measurement of high affinity bound [^{3}H]-17β-oestradiol remaining in the presence of the inhibitors. The number of inhibitors studied in cortex cytosols was therefore restricted to 6 compounds (*cis*-clomiphene, 17α-oestradiol, oestriol, oestrone, DES and unlabelled 17β-oestradiol) and each assay was carried out in duplicate so as to double the number

Table 3 Molar dissociation constants of oestrogen agonists and antagonists in reaction with cytosol high affinity receptor for 17β-oestradiol from brain, pituitary and uterus of adult metoestrus female rats

		K_{di}				
Unlabelled compound	*Order of magnitude*	*Pituitary*	*Hypothalamus*	*Amygdaloid region*	*Cortex*	*Uterus*
MER-25	10^{-8}	0.8 ± 0.1	1.0 ± 0.2	~1	—	~1
3α Diol	10^{-7}	4.1 ± 0.8*	3.4 ± 0.7*	9.8 ± 1.9	—	15.5 ± 2.8
TClo	10^{-8}	4.7 ± 0.7	8.0 ± 1.5	7.2 ± 2.1	—	7.5 ± 1.8
3β Diol	10^{-8}	4.0 ± 0.2*	3.3 ± 0.4*	5.2 ± 0.7*	—	9.8 ± 1.2
Cis Clo	10^{-8}	1.6 ± 0.9	1.3 ± 0.5	2.0 ± 0.5	1.6 ± 0.3	1.6 ± 0.8
17α E_2	10^{-9}	2.2 ± 0.2*	2.3 ± 0.1*	5.0 ± 0.8	5.2 ± 1.4	4.1 ± 0.5
E_1	10^{-9}	1.9 ± 0.1*	1.7 ± 0.1	4.3 ± 0.5	3.7 ± 0.5	3.3 ± 0.4
E_3	10^{-9}	1.6 ± 0.1	1.7 ± 0.1	2.4 ± 0.3	3.3 ± 0.9	2.1 ± 0.3
Epi E_3	10^{-9}	1.0 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	—	1.9 ± 0.4
Ru 2858	10^{-10}	3.4 ± 0.2	7.1 ± 0.7*	4.7 ± 0.7	—	4.6 ± 0.3
17β E_2	10^{-10}	1.0 ± 0.1*	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.3	1.5 ± 0.2
DES	10^{-11}	2.8 ± 0.4*	6.0 ± 0.9	4.9 ± 0.8	9.2 ± 0.8	8.9 ± 2.2

Each value is the mean (±s.e. mean) of 4 or 5 determinations.
* Significant difference from uterus $P < 0.01$.

of values contributing to the means. The results of these experiments with cytosols from metoestrus females are presented in Table 3. Values for affinity for each substance in uterine cytosol were compared with the values in cytosols from other tissues of female animals and, in paired t-tests $P < 0.01$ was taken as the criterion of significance. Significant differences from uterus were seen in 6 of the 12 compounds in pituitary and in 5 of the compounds in hypothalamus. Of these, 4 of the compounds are common to both hypothalamus and pituitary (3α androstan-diol, *trans*-clomiphene, 3β androstan-diol and 17α-oestradiol) and with a single exception (Ru 2858 in hypothalamus) the lower affinity was found in uterine cytosol. In contrast to pituitary and hypothalamus, no significant differences between the affinities in cortex and uterine cytosols were seen and there was only one compound with a different affinity in cytosol from amygdaloid region. Table 4 gives the values for the affinities estimated in cytosols from male animals. In only 3 of the 42 contrasts were the values significantly different ($P < 0.01$ in unpaired t-tests) from those in cytosols from the same tissues in females. Two of the contrasts concerned were in hypothalamus with compounds that in the female showed differential affinity between uterus and hypothalamus; the mean values for the affinities of the two compounds in male hypothalamic cytosol are remarkably close to the estimates using uterine cytosol.

Discussion

The receptors in all the tissues studied are oestrogen selective and appear to be similar but are not necessarily identical. Similarities are found in the ranking orders of the affinities of the reaction of the compounds studied with the receptors which agree well with earlier reports of specificities of receptor binding in peripheral target tissues and with the known potencies (as oestrogens or anti-oestrogens) of the compounds used (Korenman, 1969; 1970; Raynaud, 1973). Dissociation constants of the reaction between [^{3}H]-17β-oestradiol and the high affinity sites are of the order 10^{-10} M which is appropriate to receptors mediating responses at physiological plasma concentrations of the steroid (Brown-Grant, Exley & Naftolin, 1973). Binding affinities for 17β-oestradiol measured by competition did not differ significantly from the direct estimates from the binding isotherm for [^{3}H]-oestradiol.

There is, nevertheless, some significant variation seen in the affinities of the reactions in different cytosols. Before concluding from the observed differences that there is evidence for heterogeneity of oestrogen receptors, possible experimental artefacts should be considered. The presence in cytosols of endogenous inhibitors would interfere with the binding reaction, and could account for differences between tissues but this seems an improbable source of artefact since if the receptors were homogeneous this should affect all estimates of K_{di}. Similarly, the influence of errors in the estimation of unbound oestradiol (F) can be discounted. It should be noted that the calculation of K_{di} is based upon the assumption that receptor-inhibitor reaction had reached equilibrium within the incubation period, known to be adequate for the attainment of oestradiol-receptor equilibrium. The suggestion from these results of multiple forms of oestrogen receptors raises the possibility of the synthesis of tissue specific oestrogens or anti-oestrogens.

The results obtained with perphenazine and the two isomers of androstandiol are of particular interest in

Table 4 Molar dissociation constants of oestrogen agonists and antagonists in reaction with cytosol high affinity receptor for 17β-oestradiol from brain and pituitary of adult male rats

		K_{di}			
Inhibitor	*Order of magnitude*	*Pituitary*	*Hypothalamus*	*Amygdaloid region*	*Cortex*
MER-25	10^{-8}	~1	~1	~1	—
3α diol	10^{-7}	2.6±0.7	4.6±1.4	4.4±0.9	—
TClo	10^{-8}	9.3±2.1	10.2±2.9	11.2±4.5	—
3β diol	10^{-8}	3.9±0.3	3.5±0.4	5.9±0.9	—
Cis Clo	10^{-8}	1.4±0.4	2.0±0.6	3.2±1.5	2.6±1.4
17α E_2	10^{-9}	3.5±0.7	4.0±0.5*	6.0±0.7	3.9±0.6
E_1	10^{-9}	3.0±0.4	3.0±0.4*	6.0±0.9	3.7±0.7
E_3	10^{-9}	2.4±0.5	1.9±0.2	2.9±1.2	2.6±1.4
Epi E_3	10^{-9}	1.8±0.5	0.9±0.1	1.6±0.5	—
Ru 2858	10^{-10}	2.6±0.3	4.9±0.4	2.3±0.2*	—
17β E_2	10^{-10}	1.2±0.2	1.5±0.2	2.0±0.3	1.3±0.2
DES	10^{-11}	2.4±0.5	5.9±0.1	4.1±0.1	8.1±3.7

Each value is the mean (±s.e. mean) of 4–6 determinations.
* Significant difference from female $P < 0.01$.

view of the reported properties of these three compounds. Perphenazine, a lactogenic phenothiazine tranquillizer, has been reported to inhibit selectively the uptake of physiological doses of [^{3}H]-17β-oestradiol into both central and peripheral oestrogen target tissues, raising the possibility that the lactogenic effects of the drug might be mediated at least in part through binding to hypothalamic and pituitary oestrogen receptors (Shani *et al.*, 1971). In the present experiments no evidence was obtained for inhibition of [^{3}H]-17β-oestradiol binding to putative high affinity oestrogen receptor sites in any cytosol fraction, in the presence of a 5000-fold Molar excess of perphenazine. This suggests that the inhibition of [^{3}H]-17β-oestradiol uptake reported by Shani *et al.* (1971) does not represent direct interaction between perphenazine and available oestrogen receptor sites.

The ability of certain androgens, and in particular 5α androstan-3β,17β diol, to induce oestrogen-like changes in the histology of the female reproductive tract has been known for some time (Williams-Ashman & Reddi, 1972). The present results provide evidence that 5α androstan-3β,17β diol, and to a considerably lesser extent, its 3α isomer, may also interact directly with cytoplasmic oestrogen receptors, both in the uterus and in the central nervous system and the pituitary. In view of the fact that these two isomers of 5α androstandiol can be formed *in vivo* as metabolites of plasma androgen (the 3α epimer *in situ* in regions of the brain, and in the pituitary—Rommerts & van der Molen, 1971; Sholiton, Jones & Werk, 1972), these findings are of particular significance, since they suggest a mechanism, other than via aromatization to oestrogen, by which plasma androgen might impinge upon oestrogen receptor sites.

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