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A COMPARATIVE STUDY OF THE INTERACTION OF OESTRADIOL AND THE STEROIDAL PURE ANTIOESTROGEN, ICI 164,384, WITH THE MOLYBDATE-STABILIZED OESTROGEN RECEPTOR

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Summary—The kinetics of binding of oestradiol and the steroidal pure antioestrogen ICI 164,384 to the molybdate-stabilized oestrogen receptor, partially purified from pig and human uterine tissue, were determined. ICI 164,384 bound directly to the oestrogen receptor protein and the kinetic parameters of this interaction were, in general, similar to those for the binding of oestradiol, regardless of the source of the receptor protein. However, the rate of assocation of the antagonist with the receptor protein was slower when compared to that of oestradiol. Furthermore, the concentration of binding sites for the two ligands was of the same order. The binding of oestradiol resulted in a steroid–receptor complex which could be transformed *in vitro*, to a form with increased affinity for DNA–cellulose. However, the complex formed between ICI 164,384 and the receptor protein did not show increased affinity for DNA–cellulose when exposed to conditions that transformed agonist–receptor complexes. Therefore, the binding of ICI 164,384 to the oestrogen receptor protein results in a suppression of the transformation process. A similar suppression *in vivo* may account for the pure antagonist properties of ICI 164,384.

INTRODUCTION

The action of oestrogens on their target cells is mediated via the formation of a complex between the hormone and a specific receptor protein, the oestrogen receptor. Transformation of the resulting oestrogen–receptor complex to a form with increased affinity for chromatin then occurs, leading to specific genomic responses and changes in the mRNA and protein profile of target cells [1, 2].

The oestrogen receptor protein is also thought to be responsible for the mediation of the effects of antioestrogens [2, 3]. However, the molecular details of antioestrogen action remain unclear due, in part, to the absence of a high affinity antioestrogen with no oestrogenic activity, i.e. a pure antioestrogen. Conventional antioestrogens, exemplified by clomiphene and tamoxifen, are partial agonists with relatively low receptor binding affinity. For example, the affinity of the partial agonist tamoxifen for the oestrogen receptor is at least an order of magnitude less than that of oestradiol-17β [4]. The synthesis of the steroidal pure antioestrogen, ICI 164,384 [*N*-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)*N*-methylundecanamide] [3] presents an opportunity to re-examine the interaction of antagonists with the oestrogen receptor protein. A preliminary study [5], showed that ICI 164,384 does bind directly to the oestrogen receptor from human uterus but the resulting complex was not transformed *in vitro* as judged by its ability to bind to DNA–cellulose.

In the present study we report a kinetic analysis of the interaction of ICI 164,384 with the partially purified oestrogen receptor protein. This has been facilitated by the use of pig uterus, a tissue that can be obtained readily in large quantities, as source for the purification of the oestrogen receptor protein. Where possible data are compared with those obtained using the oestrogen receptor purified from human uterus, a tissue which is more difficult to obtain in good quality and quantity.

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EXPERIMENTAL

Materials

Phosphocellulose and cellulose CF11 were supplied by Whatman Biochemicals, Maidstone, England. Sephadex G-25 (PD-10 columns), CNBr-activated Sepharose CL-4B and dextran T70 were purchased from Pharmacia Fine Chemicals, London, England.

Calf thymus DNA, heparin (sodium salt, grade II) from porcine intestinal mucosa, 17β-oestradiol, diethylstilboestrol and activated charcoal were from Sigma Chemical Co., Poole, England. [2,4,6,7-^{3}H]Oestradiol (85–110 Ci/mmol), was obtained from Amersham International, Amersham, England. ICI 164,384 and [^{3}H]ICI 164,384 (47 Ci/mmol) were synthesized by ICI Pharmaceuticals, Alderley Edge, England. Dithiothreitol was from Boehringer Corporation Ltd, Lewes, England, and nylon bolting cloth (200 micron mesh) was from Simon Engineering Ltd, Stockport, England. Dichlorodimethylsilane was obtained from Aldrich Chemical Co. Ltd., Gillingham, England. All other reagents were supplied by BDH Chemicals, Poole, England, and were of the purest grade available.

Pig uteri were obtained from the local abattoir. Immediately after excision, the uteri were placed on ice, frozen as soon as possible at −20°C, and stored for up to 8 weeks. Human uteri, obtained from patients undergoing hysterectomy at local hospitals, were rapidly frozen in liquid nitrogen and stored at −70°C until required. The oestrogen receptor was purified from human uterus by methods described previously [5].

All glassware was silanized before use. DNA–cellulose [6] and heparin Sepharose [7] were synthesized according to methods described previously. Ligands were added to incubations as solutions in ethanol and the final ethanol concentration in the incubation did not exceed 5% (v/v). Control incubations contained ethanolic buffer of the same ethanol concentration as the test incubation.

Purification of the oestrogen receptor from pig uterus

All procedures were performed at 4°C. Uteri were thawed, stripped of connective tissue and cut into 3–4 mm pieces. Uterine tissue was then homogenized in 2 vol of 10 mM TEDGM buffer (10 mM sodium molybdate, 1.5 mM EDTA, 2.5 mM dithiothreitol, in 10 mM Tris/HCl, 10% (v/v) glycerol, pH 7.4 at 20°C). The homogenate was centrifuged at 105,000 *g* for 60 min; the cytosol (supernatant) was removed free of floating fat by filtering through nylon cloth and used immediately.

Solid $(NH_4)_2SO_4$ was added slowly to the cytosol, with mixing, to a final concentration of 361 g/l (60% saturation). The solution was stirred for 20 min and then the precipitated protein was collected by centrifugation at 25,000 *g* for 15 min. The supernatant was discarded and the pellets were washed gently in 10 mM TEDGM/5 mM Mg^{2+} (10 mM TEDGM containing 5 mM magnesium acetate). The precipitate was resuspended in 10 mM TEDGM/5 mM Mg^{2+} and the ionic strength adjusted to the equivalent of a 200–250 mM salt solution by the addition of 10 mM TEDGM/5 mM Mg^{2+}.

The resuspended precipitate was gently stirred for 16 h with heparin Sepharose (1 ml gel/50 ml resuspended precipitate). The suspension was then filtered, under vacuum, on a glass-sintered funnel until a moist gel was obtained. The gel was washed with 10 mM TEDGM/5 mM Mg^{2+}, resuspended in a small volume of this buffer and poured into a column. The column was then washed with the same buffer until the eluate was protein free; bound protein was eluted with heparin, 5 mg/ml in 10 mM TEDGM buffer. This fraction contained the oestrogen receptor protein purified 50–60-fold over the cytosolic fraction.

Binding assays

Heparin-eluted protein from the heparin Sepharose column was incubated with a range of concentrations of [^{3}H]oestradiol and [^{3}H]ICI 164,384 for 24 h at 0°C in the presence and in the absence of a 100-fold molar excess diethylstilboestrol. Ligand not bound to protein was removed by the addition of one volume of dextran-charcoal (2% (w/v) Norit A-activated charcoal, 0.2% (w/v) dextran T70 in 10 mM TEGM buffer). After incubation on ice for 20 min charcoal was removed by centrifugation and 0.2 ml of the supernatants removed for scintillation counting.

Relative binding affinity

The relative binding affinity of ICI 164,384 for the oestrogen receptor was determined using a modification of the method described by Wakeling and Slater [8]. Oestrogen receptor preparations were incubated with 10 nM [^{3}H]oestradiol plus various concentrations of ICI 164,384 or oestradiol (0–200 nM) in the presence and in the absence of 1 μM diethylstilboestrol. After incubation for 24 h at 0°C or for 2 h at 25°C, protein-bound steroid was determined by the dextran-charcoal competitive binding assay described above. Graphs were prepared of the ratio of the amount of [^{3}H]oestradiol bound in the absence of antagonist to the amount of [^{3}H]oestradiol bound in the presence of oestradiol or antagonist against the concentration of competing ligand. The relative affinity of the receptor for the antagonist compared to oestradiol was then obtained from the ratio of the gradients of the plots.

Association rate constant (K_{+1}) at 0°C

Partially purified oestrogen receptor, prepared as described above, was incubated with 2 nM [^{3}H]oestradiol or 2 nM [^{3}H]ICI 164,384. Non-specific binding was measured in parallel incubations containing the labelled ligands and 200 nM diethylstilboestrol. At various times aliquots were removed and incubated with one volume of dextran-charcoal suspension to stop the reaction before centrifugation and determination of the radioactivity present in the supernatant. The data were plotted in the form ln [(Ro-So)/(So-SR)]/(Ro-So) against t where So is the total concentration of tritiated ligand and SR the concentration of specifically bound tritiated ligand at time t. The gradient of the resulting straight line equals the association constant, K_{+1}. The total

amount of bound receptor was measured after 18 h with saturating amounts of [^{3}H]oestradiol.

Dissociation rate constant (K_{-1})

Samples of partially purified oestrogen receptor were incubated with either 4 nM [^{3}H]oestradiol or 4 nM [^{3}H]ICI 164,384 in the presence and absence of 400 nM diethylstilboestrol for 16 h at 0°C. Free ligand was then removed by chromatography on small columns of Sephadex G-25 (bed vol 9.1 ml) and unlabelled 1 μM oestradiol added to prevent reassociation of dissociating labelled ligand. Eluates from the Sephadex G-25 columns were then incubated at 0 or 10°C and samples removed at various times for the measurement of protein-bound radioactivity. The stability of ligand–receptor complexes at 0 and 10°C was assessed by omitting both chromatography on Sephadex G-25 and addition of 1 μM oestradiol.

Assessment of transformation status of steroid–receptor complexes

Transformation of preparations of steroid–receptor complexes was determined using a modification of the method of Kalimi *et al.* [9]. Ligand–receptor complexes were prepared by the incubation of partially purified receptor with 4 nM tritiated steroid in the presence and absence of 400 nM diethylstilboestrol for 16 h at 0°C. Molybdate was then removed by chromatography on Pharmacia PD-10 columns equilibrated with 10 mM TEDG (10 mM TEDGM excluding molybdate). Tritiated steroid (4 nM) or 4 nM tritiated steroid plus 400 nM diethylstilboestrol was reintroduced to the appropriate incubations and each incubation divided. One half of each incubation was transformed by incubation at 25°C for 30 min whilst the other half was incubated at 0°C for 30 min. The process of transformation was stopped by cooling the incubations on ice for 3 min. Free steroid was then removed, and molybdate added, by chromatography of each incubation on Pharmacia PD-10 columns equilibrated with 10 mM TEDGM.

Increasing amounts of steroid–receptor complex in a total volume of 0.1 ml were incubated with 0.4 ml DNA–cellulose for 30 min at room temperature with shaking every 5 min. After 30 min 2 ml buffer were added, incubations were mixed and centrifuged at 600 *g* for 5 min. The supernatant was discarded and the washing procedure repeated twice. The final DNA–cellulose pellet was resuspended in 1 ml buffer and bound radioactivity determined by liquid-scintillation counting.

RESULTS

Parameters for the binding of [^{3}H]oestradiol and [^{3}H]ICI 164,384 to the oestrogen receptor

The binding of oestradiol and ICI 164,384 to the molybdate-stabilized oestrogen-receptor was assessed by incubating partially purified oestrogen receptor protein, eluted from the heparin Sepharose column by heparin, with a range of concentrations of each of the tritiated ligands for 24 h at 0°C in the presence and in the absence of a 100-fold molar excess diethylstilboestrol. After separation of free ligand, protein-bound radioactivity was measured and the data analysed by the method of Scatchard [10]. The equilibrium dissociation constants, (K_d), for the interaction of both ligands at 0°C with the receptor purified from pig uterus were found to be of the same order; 0.90 ± 0.20 nM for [^{3}H]oestradiol and 1.91 ± 0.19 nM for [^{3}H]ICI 164,384 (Table 1 and Fig. 1). The concentration of receptor binding sites for the agonist and antagonist was 2807 ± 1186 and 3127 ± 1103 fmol/mg respectively (Table 1 and Fig. 1). The large standard deviations were due to the wide range of receptor concentrations observed, however, for any one receptor preparation the concentration of receptor binding sites was similar for both ligands. The equilibrium dissociation constant for the interaction of [^{3}H]oestradiol with unfractionated cytosolic receptor preparations from pig uterine tissue was 1.6 nM (data not shown).

The equilibrium dissociation constants for the interaction of the two ligands with the oestrogen

Table 1. Comparison of the physicochemical characteristics of the interaction of [^{3}H]oestradiol and [^{3}H]ICI 164,384 with the oestrogen receptor protein from pig and human uterus

Parameter		[^{3}H]oestradiol Pig	[^{3}H]oestradiol Human	[^{3}H]ICI 164,384 Pig	[^{3}H]ICI 164,384 Human
Equilibrium dissociation constant (nM)		0.90 ± 0.20	0.44 ± 0.20	1.91 ± 0.19	0.69 ± 0.10
Number of binding sites		2807 ± 1186 (1655–4026)	1360 ± 1181 (362–2665)	3127 ± 1103 (1893–4017)	1037 ± 916 (456–2094)
Relative binding affinity:					
Cytosolic receptor	0°C	100	100	5.9 ± 1.0	ND
	25°C	100	100	22.9 ± 5.1	17.0 ± 4.2
Purified receptor	0°C	100	100	40.7 ± 9.1	ND
	25°C	100	100	154 ± 48	ND
Association rate constant ($nM^{-1}\ s^{-1}$)		2.72×10^{-4} $\pm 0.63 \times 10^{-4}$	ND	3.03×10^{-5} $\pm 0.75 \times 10^{-5}$	5.00×10^{-5}*
Dissociation rate constant (min^{-1})	0°C	4.28×10^{-4} $\pm 2.06 \times 10^{-4}$	ND	2.92×10^{-4} $\pm 1.10 \times 10^{-4}$	ND
	10°C	1.51×10^{-3} $\pm 0.24 \times 10^{-3}$	5.97×10^{-4} $\pm 0.94 \times 10^{-4}$	8.01×10^{-4} $\pm 1.20 \times 10^{-4}$	1.49×10^{-3}*

Results are expressed as mean ± SD of at least three determinations except where indicated by an asterisk. ND, not determined. Figures in parentheses indicate ranges.

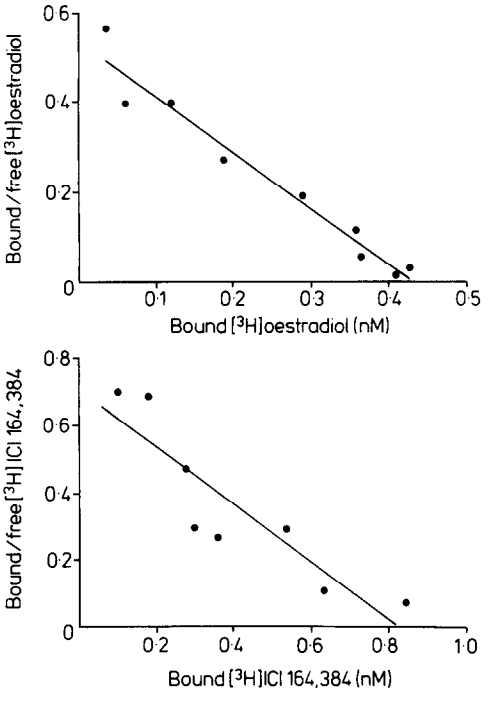

Fig. 1. Scatchard plots of the binding of (a) [^{3}H]oestradiol and (b) [^{3}H]ICI 164,384 to the partially purified pig uterine oestrogen receptor. Preparations of oestrogen receptor protein were incubated with various concentrations of ligand in the presence and in the absence of 100-fold molar excess diethylstilboestrol at 0°C for 24 h. Free steroid was then removed by incubation with dextran-coated charcoal. It should be noted that the results were not obtained using the same preparation of receptor.

receptor purified from human uterus at 0°C were also of the same order and furthermore, were comparable to those obtained using receptor protein purified from pig uterus. The constants for the interaction of [^{3}H]oestradiol and [^{3}H]ICI 164,384 were 0.44 ± 0.20 and 0.69 ± 0.10 nM respectively. The concentration of receptor binding sites was also similar (Table 1 and Fig. 2).

The partial purification of the oestrogen receptor protein from both pig and human uterine tissue resulted in preparations in which the non-specific binding of [^{3}H]oestradiol was less than 5% of the total binding for this ligand and that for [^{3}H]ICI 164,384 was less than 50% of the total binding for this antagonist.

Relative binding affinity of [^{3}H]oestradiol and [^{3}H]ICI 164,384 for the oestrogen receptor

To evaluate the affinity of the oestrogen receptor for ICI 164,384, receptor preparations were incubated with [^{3}H]oestradiol and, concurrently, with increasing concentrations of either unlabelled oestradiol or ICI 164,384 at 0°C for 24 h or 25°C for 2 h before removal of free steroid by incubation with dextran-coated charcoal. Figures 3 and 4 show that ICI 164,384 competes with oestradiol for binding sites on the receptor protein from pig uterus and that the binding affinity of ICI 164,384 is reduced compared with that of oestradiol. The relative binding affinity of ICI 164,384 for the cytosolic oestrogen receptor protein was $5.9 \pm 1.0\%$ that of oestradiol at 0°C (Fig. 3a and Table 1), increasing to $22.9 \pm 5.1\%$ at 25°C (Fig. 3b and Table 1). However, the relative binding affinity of the antagonist was $40.7 \pm 9.1\%$ that of oestradiol at 0°C, increasing to $154 \pm 48\%$ at 25°C, when partially purified preparations of the oestrogen receptor protein from pig uterus were used (Figs 4a and b, and Table 1). The relative binding affinity of [^{3}H]ICI 164,384 for the cytosolic oestrogen receptor from human uterus was $17.0 \pm 4.2\%$ of that of oestradiol at 25°C (Table 1) and was, therefore, of the same order as that observed using cytosolic oestrogen receptor from pig uterus.

The association of ICI 164,384 with the partially purified oestrogen receptor at 0°C

Having established that the receptor has a lower affinity for ICI 164,384 than oestradiol at 0°C we investigated whether this was due to a decreased association rate, an increased dissociation rate, or both. Figure 5 shows that the association rate of [^{3}H]ICI 164,384 to the receptor protein purified from pig uterus is reduced compared with oestradiol. The

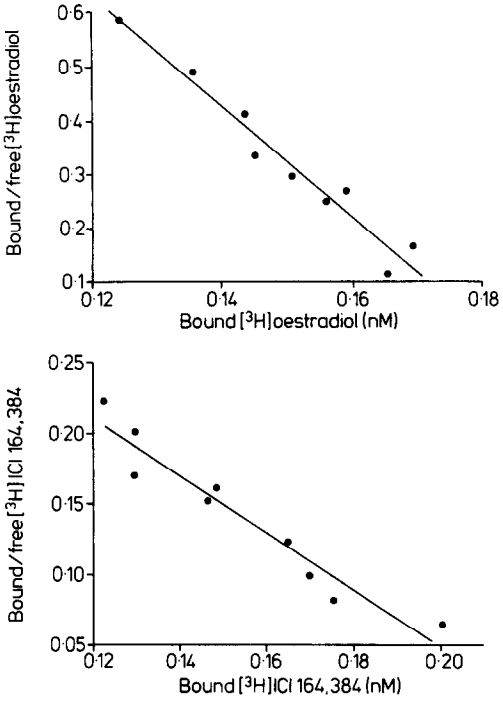

Fig. 2. Scatchard plots of the binding of (a) [^{3}H]oestradiol and (b) [^{3}H]ICI 164,384 to the partially purified human uterine oestrogen receptor. Preparations of oestrogen receptor protein were incubated with various concentrations of ligand in the presence and in the absence of 100-fold molar excess diethylstilboestrol at 0°C for 24 h. Free steroid was then removed by incubation with dextran-coated charcoal. It should be noted that the results were not obtained using the same preparation of receptor.

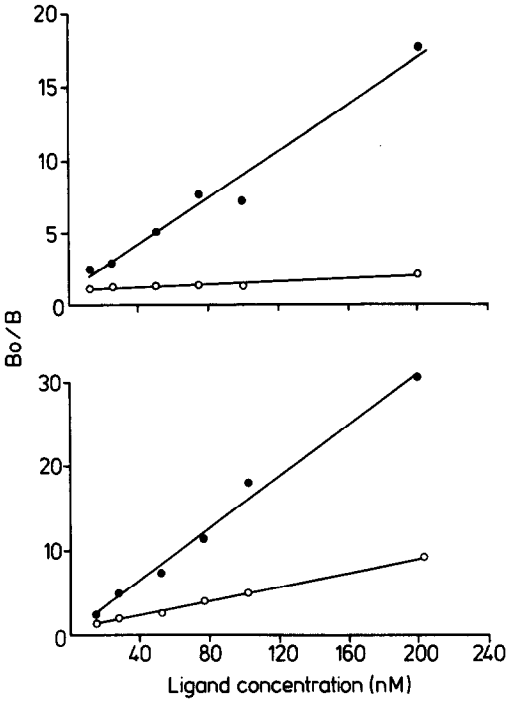

Fig. 3. Competitive inhibition of [^{3}H]oestradiol binding. Cytosol, prepared from pig uterine tissue, was incubated for (a) 24 h at 0°C or (b) 2 h at 25°C with 10 nM [^{3}H]oestradiol and the indicated concentrations of oestradiol (●—●) or ICI 164,384 (○—○) in the presence and absence of 1 μM diethylstilboestrol. Values for specific binding in the presence of competitors (B) were normalized relative to control values (B_0).

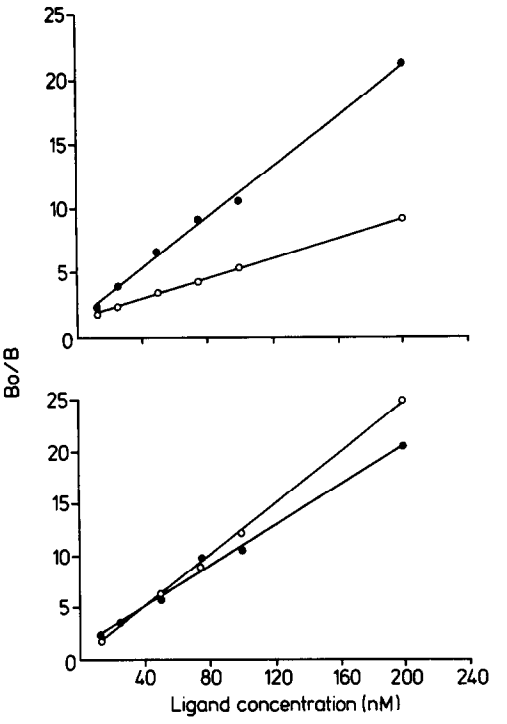

Fig. 4. Competitive inhibition of [^{3}H]oestradiol binding. Partially purified preparations of the pig uterine oestrogen receptor were incubated for (a) 24 h at 0°C or (b) 2 h at 25°C with 10 nM [^{3}H]oestradiol and the indicated concentrations of oestradiol (●—●) or ICI 164,384 (○—○) in the presence and absence of 1 μM diethylstilboestrol. Values for specific binding in the presence of competitors (B) were normalized relative to control values (B_0).

association rate constant for the interaction of [^{3}H]oestradiol with the receptor protein was $2.72 \times 10^{-4} \pm 0.63 \times 10^{-4}$ nM^{-1} s^{-1} whilst that for [^{3}H]ICI 164,384 was found to be $3.03 \times 10^{-5} \pm 0.75 \times 10^{-5}$ nM^{-1} s^{-1}. A similar rate of association was observed for the interaction of the antagonist with the oestrogen receptor protein purified from human uterine tissue (Table 1 only one result obtained). Therefore, the reduced relative binding affinity of the receptor protein for ICI 164,384 at 0°C may, to some degree, be a reflection of a reduced association rate.

In a single determination of the association rate of oestradiol with the cytosolic receptor protein from pig uterus, a value of 2.66×10^{-4} nM^{-1} s^{-1} was obtained. This is a similar rate to that observed for oestradiol with the partially purified receptor from pig uterus (Table 1).

The dissociation of ICI 164,384 from the antagonist–receptor complex

The dissociation rates of both [^{3}H]oestradiol and [^{3}H]ICI 164,384 from their respective steroid–receptor complexes at 0°C were extremely slow and thus only approximate estimates of these rates were obtained (Table 1). The rate of dissociation of the [^{3}H]ICI 164,384–receptor complex was therefore

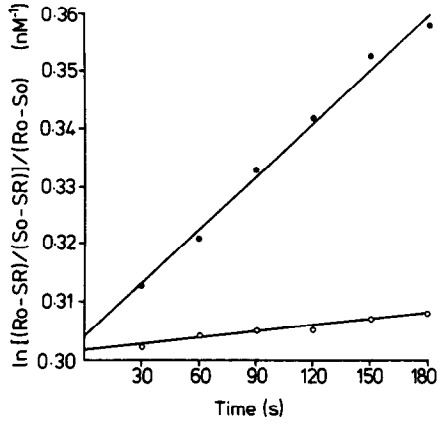

Fig. 5. Determination of association rate constant for [^{3}H]ICI 164,384. Partially purified preparations of the pig uterine oestrogen receptor were incubated with either 2 nM [^{3}H]oestradiol (●—●) or 2 nM [^{3}H]ICI 164,384 (○—○) in the presence and in the absence of 200 nM diethylstilboestrol. Specific binding was determined by dextran-coated charcoal adsorption at the indicated times. The data were plotted as described in the Experimental section. Total oestrogen receptor concentration was determined in a parallel incubation containing 10 nM [^{3}H]oestradiol incubated for 18 h at 0°C.

determined at 10°C and compared with that of the [^{3}H]oestradiol–receptor complex, using complexes formed between the partially purified receptor and the antagonist or agonist. The rate constant for the dissociation of the antagonist from complexes formed with the receptor protein from pig uterus was approximately twice that of [^{3}H]oestradiol (Fig. 6 and Table 1). The $t_{1/2}$ for [^{3}H]ICI 164,384 was 879 ± 128 min which corresponds to a dissociation rate constant of $8.01 \times 10^{-4} \pm 1.20 \times 10^{-4}$ min^{-1} whilst the corresponding values for the dissociation of [^{3}H]oestradiol were 469 ± 72.2 min and $1.51 \times 10^{-3} \pm 0.24 \times 10^{-3}$ min^{-1}, respectively. The same relationship between the dissociation of the two ligands was obtained at 0°C (Table 1). Both oestrogen and antioestrogen occupied receptor complexes were stable throughout the duration of the experiment. The dissociation rate constants for the dissociation of either ligand from the partially purified receptor from human uterus were comparable to those obtained using receptor from pig uterus (Table 1).

Transformation of steroid–receptor complexes

The transformation of steroid–receptor complexes, after exposure to increased temperature in the absence of molybdate, is reflected by the extent of binding of the complex to DNA–cellulose. To investigate the ability of agonist– and antagonist–receptor complexes to undergo transformation, partially purified receptor from both pig and human uterine tissue was incubated at 0°C for 16 h with 10 nM [^{3}H]oestradiol or 10 nM [^{3}H]ICI 164,384 in the presence and in the absence of 1.0 μM diethylstilboestrol. Molybdate was removed and samples were then incubated at 0 or 25°C for 30 min before being assayed for the extent of transformation. The degree of transformation was assessed by subtracting the non-specific binding from the total binding and the resulting specific binding observed at 0°C from that at 25°C for each steroid.

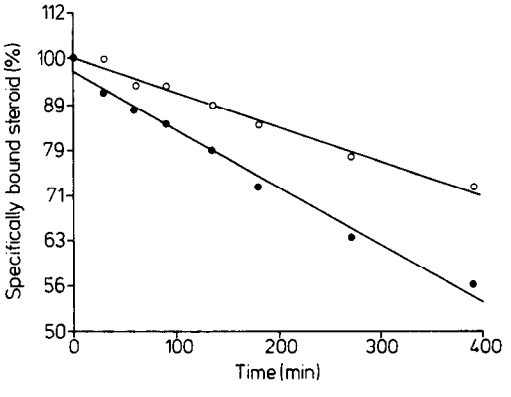

Fig. 6. Dissociation of [^{3}H]oestradiol (●—●) or [^{3}H]ICI 164,384 (○—○) from the partially purified pig uterine oestrogen receptor at 10°C. Partially purified oestrogen receptor protein was incubated at 0°C for 16 h with 4 nM [^{3}H]oestradiol or 4 nM [^{3}H]ICI 164,384 in the presence and in the absence of 400 nM diethylstilboestrol. Unbound ligand was removed by chromatography on Sephadex G-25 columns and all incubation tubes were adjusted to 1 μM oestradiol. Bound radioactivity was determined at the indicated times by dextran-coated charcoal adsorption.

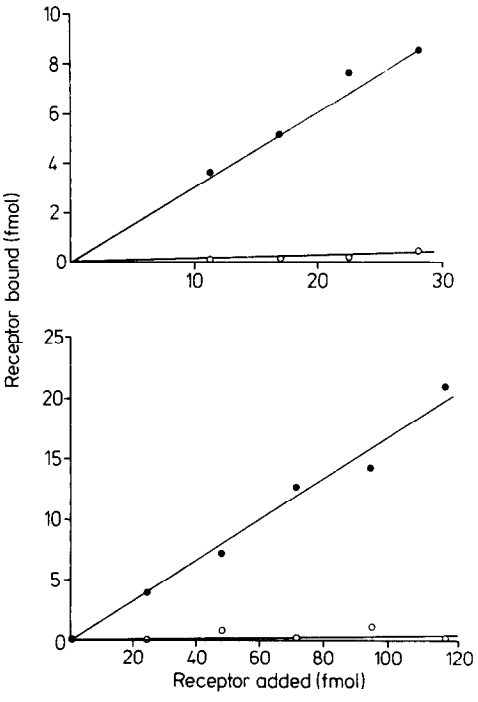

Fig. 7. Binding of transformed [^{3}H]oestradiol–receptor complexes and [^{3}H]ICI 164,384–receptor complexes to DNA–cellulose. Increasing amounts of [^{3}H]oestradiol–receptor complex (●—●) or [^{3}H]ICI 164,384 complex (○—○) from (a) human uterus and (b) pig uterus were incubated, before and after transformation, with DNA–cellulose for 30 min before the extent of binding to the matrix was measured by scintillation counting. For full experimental details see text.

The affinity of complexes formed between [^{3}H]ICI 164,384 and the receptor protein for DNA–cellulose did not increase on exposure to conditions which increased significantly the affinity of [^{3}H]oestradiol–receptor complexes for the same matrix (Fig. 7). The amount of antagonist–receptor complex bound to DNA–cellulose was approximately 2% of the amount of agonist–receptor complex bound to the matrix after exposure to transforming conditions regardless of the source of the oestrogen receptor protein.

DISCUSSION

Until recently, all available antioestrogens possessed some degree of agonist activity. This activity has restricted the range of potential therapeutic applications in the treatment of oestrogen-responsive diseases, and has also limited their use in studies of the molecular mechanisms underlying oestrogen and antioestrogen action. However, it has been shown that 7α-alkyl amide analogues of oestradiol behave as pure oestrogen antagonists in the rat and

mouse [3, 11 and 12]. We have studied the interaction of one of these compounds, the steroidal, pure antioestrogen ICI 164,384, with the oestrogen receptor.

One significant practical problem associated with the study of the mechanism of action of antioestrogens is that they are, generally, extremely hydrophobic [13]. This hydrophobicity leads to a very high level of non-specific binding of the ligand when cytosolic preparations of tissues are used as the source of oestrogen receptor protein. For [^{3}H]ICI 164,384 non-specific binding was at least 95% of the total binding in such impure preparations. Partial purification of the oestrogen receptor from both pig and human uterine tissue resulted in receptor preparations in which the non-specific binding of [^{3}H]ICI 164,384 was less than 50% of the total binding for this ligand. Purification did not lead to any major change in the nature of the protein present in pig uterus since the equilibrium dissociation constant for the interaction of receptor protein with [^{3}H]oestradiol did not change whether unfractionated cytosolic preparations or heparin-eluted protein from the heparin Sepharose column was used as the source of oestrogen receptor. The pure antioestrogen bound to the same number of sites on the receptor protein as oestradiol with an affinity comparable with that of oestradiol. This was the case for the interaction of the antagonist with the receptor protein purified from either pig or human tissue. Furthermore, the K_d for the interaction of the two ligands with the human receptor protein was of the same order as that for the interaction of these ligands with the receptor protein from pig uterus.

The relative affinity of ICI 164,384 for the oestrogen receptor was less than that of oestradiol when cytosolic preparations were used as the source of the receptor protein. As stated above, the non-specific binding of the antagonist is extremely high in such preparations and this would effectively reduce the amount of ICI 164,384, and therefore the actual concentration of this ligand, available for interaction with the receptor protein. An increase in the relative affinity of the antagonist for the oestrogen receptor protein was seen after purification of the receptor protein, a process which reduced the non-specific binding. The relative affinity of ICI 164,384 for the receptor protein also increased when measured at 25°C compared to that measured at 0°C. An increase in temperature could also decrease non-specific binding due to non-specific hydrophobic interactions, occurring as a consequence of the highly hydrophobic nature of the antagonist molecule, increasing the relative affinity of ICI 164,384 for the oestrogen receptor at 25°C compared with 0°C. Scatchard analyses showed that the equilibrium dissociation constants for the interactions of [^{3}H]oestradiol and [^{3}H]ICI 164,384 with the receptor are of the same order suggesting that the affinities of the two ligands for the receptor are comparable when measured under equilibrium conditions in the absence of competing ligand of different hydrophobicity.

The dissociation rates of both [^{3}H]oestradiol and [^{3}H]ICI 164,384 from the respective steroid–receptor complexes were very slow at 0°C. Indeed, rates were so slow that only an approximate estimate of the dissociation rate constants could be made. However, at 10°C, the rate of dissociation of oestradiol from the oestradiol–receptor complex was approximately twice that of ICI 164,384 from the antioestrogen–receptor complex. A similar relationship was suggested by a comparison of the estimated values obtained at 0°C. This fulfils an important criterion for potent antioestrogenic activity, namely that once bound to the receptor protein the ligand does not dissociate rapidly but rather dissociates at a rate equivalent to that of oestradiol. In this respect ICI 164,384 is similar to 4-hydroxytamoxifen [8, 13] and emphasises that, for both steroidal and nonsteroidal antioestrogens, a rapid rate of dissociation is not diagnostic of antagonist activity as claimed previously [14].

As the equilibrium constants and the rates of dissociation of the two ligands were of the same order it was anticipated that the rates of association were also of the same order. This was not found to be the case with the rate of association of [^{3}H]ICI 164,384 being 10-fold slower than that of [^{3}H]oestradiol at 0°C. This slower rate of association of ICI 164,384 with the oestrogen receptor protein may be a reflection of the large, bulky 7α-side chain present in ICI 164,384 but not oestradiol [3]. This may cause steric hindrance of antagonist binding thus resulting in a reduced rate of association compared with that of oestradiol. However, this reduction in the rate of association of the antagonist, compared with that of the agonist, is not so great as to prevent its use for the study of antagonist–receptor interactions.

The association rates obtained for the interaction of oestradiol with both the cytosolic and the partially purified receptor from pig uterus, 2.66×10^{-4} $nM^{-1}\,s^{-1}$ and $2.72 \times 10^{-4}\,nM^{-1}\,s^{-1}$ respectively, are in good agreement with values previously reported for the association of this ligand with the receptor in cytosolic preparations of calf uterus and immature rat uterus [15, 16]. Furthermore, purification of the protein did not affect this interaction. It was not possible to calculate a theoretical equilibrium dissociation constant from the results obtained for the association and dissociation rates as the experiments were performed at different temperatures.

Since the antioestrogen ICI 164,384 binds to the oestrogen receptor with kinetic characteristics similar to those of oestradiol then, for ICI 164,384 to display antioestrogenic properties, differences must exist between the post-binding events occurring on the binding of agonist or antagonist. The binding of the antiglucocorticoid RU38486 to the glucocorticoid hormone receptor has been shown to prevent the process of transformation of the glucocorticoid

receptor protein, thereby preventing the stimulation of transcription [15]. Traditionally, receptor transformation has been measured by the increased binding of ligand–receptor complexes to DNA–cellulose after exposure to conditions known to cause transformation. The binding of ICI 164,384 to the oestrogen receptor molecule purified from either pig or human uterus, prevents the process of transformation. However, the ICI 164,384–receptor complex can be located in the nuclear fraction after fractionation of cells exposed to transforming conditions in the presence of antioestrogen (Nicholson, unpublished data). Therefore, it is important to note that the binding of ligand–receptor complex to DNA–cellulose simply demonstrates whether or not the complex is able to undergo the transformation process. The binding of ICI 164,384 to the oestrogen receptor protein does not prevent the intra-molecular changes required to alter the localization of the antioestrogen–receptor complex within the nucleus but does appear to prevent those changes necessary for the complex to bind to DNA and thereby initiate transcription. This is consistent with the hypothesis of Duax and Griffin, that ICI 164,384 acts as an active rather than a passive antagonist [18].

Thus, the steroidal pure antioestrogen, ICI 164,384, interacts directly with the oestrogen receptor from both pig and human uterus. The kinetics of the interaction are, in general, similar to those for the interaction of oestradiol with the receptor protein. However, the association rate observed for the interaction of [^{3}H]ICI 164,384 with the receptor protein was 10-fold slower than that of oestradiol. Binding of the antioestrogen to the hormone receptor protein results in a complex which does not undergo transformation as judged by an increased affinity for DNA–cellulose. The latter property is the most important biochemical criterion defining a pure antioestrogen. Therefore, this compound represents the first molecule suitable for use in definitive studies of the molecular mechanisms underlying antioestrogen action, and represents a class of molecules with improved antioestrogenic activity which may have wide therapeutic applications.

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