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The Stability of the Uterine Estrogen Receptor When Complexed with Estrogens or Antiestrogens

EDWARD J. PAVLIK[1] AND BENITA S. KATZENELLENBOGEN

Department of Physiology and Biophysics, University of Illinois, and School of Basic Medical Sciences, University of Illinois College of Medicine, Urbana, Illinois 61801

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SUMMARY

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As probes for possible conformational differences in estrogen receptor complexes when liganded with estrogen agonists or estrogen antagonists (antiestrogens), we have examined ligand-mediated thermal stability and resistance to trypsin-mediated proteolysis of uterine receptors occupied by a variety of steroidal and nonsteroidal estrogens and antiestrogens displaying a wide range of binding affinities for receptor. The estrogens examined (with chemical names and relative binding affinities in parentheses) were diethylstilbestrol (α,α'-diethyl-4,4'-stilbenediol (126%)), estradiol-17β (estra-1,3,5(10)-triene-3,17β-diol (100%)), 11β-methoxy-17-α-ethynylestradiol, R2858 (11β-methoxy-17α-ethynyl-estra-1,3,5(10)triene-3,17β-diol (65%)), zearalanol, P-1496 (6-(6,10-dihydroxyundecyl)-β-resorcylic acid μ-lactone (14%)), estriol (estra-1,3,5(10)-triene-3,16α,17β-triol (19%)), estrone (3-hydroxy-estra-1,3,5(10)-triene-17-one (15%)), and estradiol-17α (estra-1,3,5(10)-triene-3,17α-diol (5%)); the antiestrogens examined were CI-628 demethylated (α-[4-pyrrolidinoethoxy]phenyl4-hydroxy-α'-nitrostilbene (70%)), CI-628 (α-[4-pyrrolidinoethoxy]-phenyl-4-methoxy-α'-nitrostilbene (5%)), 11α-methoxyethynylestradiol, RU16117 (11α-methoxy-17α-ethynylestra-1,3,5(10)-triene-3,17β-diol (4%)), tamoxifen (*trans*-1 (*p*-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene (5%)), and U23,469 ((±)-*cis*-3-[*p*-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol (0.3%)). Ligand-mediated thermal stability and resistance to tryptic proteolysis were observed to be closely related to binding affinity for receptor and not to whether the ligand was an agonist or antagonist. Consequently, while the receptor may distinguish an agonist from an antagonist by some resulting conformational perturbation that is ultimately translated into different biological responses, this change is not manifested in any characteristic fashion by ligand-mediated thermal stability or protection against tryptic proteolysis.

INTRODUCTION

The class of pharmacological compounds broadly recognized as antiestrogens (or antagonists to estrogens) is generally typified by their ability to prevent estrogens from expressing their full biological effects in estrogen target tissues. Some of the biological responses antagonized by antiestrogens are uterine growth and the growth of hormone-dependent mammary tumors; these compounds also stimulate pituitary gonadotropin output by antagonizing estrogen feedback at the hypothalamic level in women (1, 2). In terms of the molecular aspects of antiestrogen action, experimental evidence suggests that antiestrogens exert their inhibitions by competing with estrogens for the binding site on the cytoplasmic estrogen receptor; that they influence the nature and duration of the association between receptors and chromatin binding sites; and that they alter the regeneration of the cytoplasmic receptor (1–10). It is tacitly supposed that some difference in the physical interaction between antiestrogen and receptor must underly the difference between ligand-mediated agonism and antagonism.

Recently, Capony and Rochefort (11) have suggested that as an antagonist, tamoxifen is unable to protect the estrogen receptor against thermal inactivation and, consequently, this inability may reflect the failure of antagonists to induce or maintain a conformation of the recep-

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[1] Present address: Department of Obstetrics and Gynecology, University of Kentucky, Lexington, Kentucky 40506.

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tor protein which can be promoted by agonists. In trying to document how the receptor might distinguish between an agonist and an antagonist, we have examined a number of radiolabeled estrogens and antiestrogens with different binding affinities in terms of their ability to stabilize the estrogen receptor against heat inactivation and to protect the receptor against proteolysis by trypsin. While we have found that different ligands vary widely in the degree of stability and protection that they afford, their effectiveness parallels their binding affinity for receptor and not their agonist/antagonist character.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats were obtained from the Holtzman Company (Madison, Wisconsin) and used at 19–21 days of age. They received food and water ad libitum and were maintained under controlled lighting conditions in an air-conditioned facility.

Radiolabeled estrogens and antiestrogens. The following tritium-labeled estrogens were obtained from Amersham Corp.: [2,4,6,7-^{3}H]estradiol-17β (108 Ci/mmol), [2,4(N)-^{3}H]estradiol-17α (44.7 Ci/mmol), [2,4,6,7(N)-^{3}H]estriol (106 Ci/mmol), [2,4,6,7-^{3}H]estrone (102 Ci/mmol), and [monoethyl-^{3}H]diethylstilbestrol (81 Ci/mmol). Tritium-labeled moxestrol ([11β-methoxy-^{3}H]R2858 (53 Ci/mmol)) and [11α-methoxy-^{3}H]RU16117 (48 Ci/mmol) were generously provided by Dr. Jean-Pierre Raynaud of Roussel-UCLAF, Romainville, France. Tritiated tamoxifen (19 Ci/mmol) was provided by ICI Americas, Inc. (Wilmington, Delaware), and ^{3}H-P1496 (30 Ci/mmol; Ref. 12) by the IMC Chemical Corp. (Terre Haute, Indiana). The following radiolabeled compounds were prepared in the laboratory of Dr. John Katzenellenbogen, Department of Chemistry, University of Illinois, as described: [^{3}H]CI-628 (16 Ci/mmol (13)), [^{3}H]CI-628M (22 Ci/mmol (14)), and [^{3}H]U23469 (16 Ci/mmol (15)). Chemical structures, names, and abbreviations of these compounds are presented in Fig. 1. The purity of all compounds was checked by thin-layer silica gel chromatography in benzene–ethanol (95:5, v/v) containing 1% triethylamine.

Preparation of cytosol and determination of specific binding to the estrogen receptor. Uteri were homogenized (2 uteri/ml) in 10 mM Tris–HCl buffer (pH 7.4) at 0–4°C. A high-speed supernatant was obtained by centrifugation at 180,000*g* for 45 min (0–4°C) in a Beckman ultracentrifuge. This high-speed supernatant, referred to here as the estrogen receptor-containing "cytosol," was treated with a 5% slurry of dextran-coated charcoal (5 g charcoal, 0.5 g dextran C/100 ml buffer; slurry:cytosol, 1:9 v/v) as previously described (3, 16) in order to remove any endogenous steroids that might be present in trace concentrations. This steroid-stripped cytosol was then diluted to 1 uterine equivalent/ml and incubated with the appropriate radiolabeled ligand (10 nM, with and without 2 μM DES) for 16–20 h at 0–4°C to fill estrogen receptor sites. Bound ligand was determined after dextran-coated charcoal adsorption of free ligand as described previously (3, 16). Binding of [^{3}H]estradiol or other tritiated ligands by the estrogen receptor was estimated as the difference between the total binding of [^{3}H]-estradiol alone and the low-affinity, nonsaturable binding determined in the presence of [^{3}H]estradiol and a 200-fold excess of nonradioactive diethylstilbestrol (DES) as previously reported (17). This difference, or the binding which is competable by excess DES, is referred to as "specific binding" by the estrogen receptor (3, 13, 16). Liquid scintillation counting was performed in a Triton X-114–xylene fluor with correction for quench made on each sample (3, 13, 16). All ligands described in this report have been shown to interact with the estrogen receptor as determined by sucrose gradient analysis and by their ability to specifically compete with [^{3}H]estradiol for binding to the estrogen receptor.

Determination of the relative binding affinity (RBA). The relative binding affinity for each ligand was determined as previously described (12) and was taken as the molar ratio of nonradioactive ligand to nonradioactive estradiol-17β which inhibited 50% of the specific [^{3}H]-estradiol binding. These estimates were confirmed for the radiolabeled compounds by determining the specific binding which resulted after charging at the K_d for estradiol (0.2 nM) and normalizing this binding to the amount of estradiol specific binding.

Assays for thermal stability and tryptic proteolysis. Cytosols charged with the appropriate ligands ± excess DES for 16–20 h at 0–4°C were incubated at 30°C for varying periods of time; the reaction was stopped by cooling on ice. Tryptic proteolysis was performed at 30°C using trypsin (Type XI, Sigma) at a final concentration of 30 μg/ml with cytosol at 1 uterine equivalent/ml. The tryptic proteolysis was stopped by cooling on ice and by the addition of soybean trypsin inhibitor (Type 11S obtained from Sigma; 2.5 μg/μg trypsin). Samples were then treated with dextran-coated charcoal as previously described to remove free ligand and aliquots were counted in Triton-X-114–xylene scintillation fluid to measure receptor–ligand complexes.

Because the nonspecific binding varies with different ligands, it was necessary to perform these experiments with ligand concentrations adjusted so that nonspecific binding for any ligand would be no greater than 60% of the total binding. The upper limit of ligand concentration, which would allow the precise determination of specific binding, was determined to be 10 nM ligand, which is 50 times in excess of the K_d for $E_2$17β (0.2 nM). At this ligand concentration, more than 90% of the estradiol-17β-specific binding sites were saturated by DES, CI-628M, estrone, estriol, P-1496, and estradiol-17α; ~75% of the estradiol-specific sites were saturated by R2858; 30–40% of these sites were saturated by CI-628 and RU16117, while 15–20% of the estradiol-specific sites were saturated by U23 and tamoxifen.

In the case of the low-affinity ligands which do not fully saturate receptors at 10 nM, the fraction of receptor that is unoccupied will be disappearing quite rapidly. We have constructed our binding assays so that this effect is minimized, since only the decay in binding of those sites filled with ligand is followed. When the receptor is only partially saturated with [^{3}H]estradiol, the thermal stability and trypsin resistance of the occupied sites are

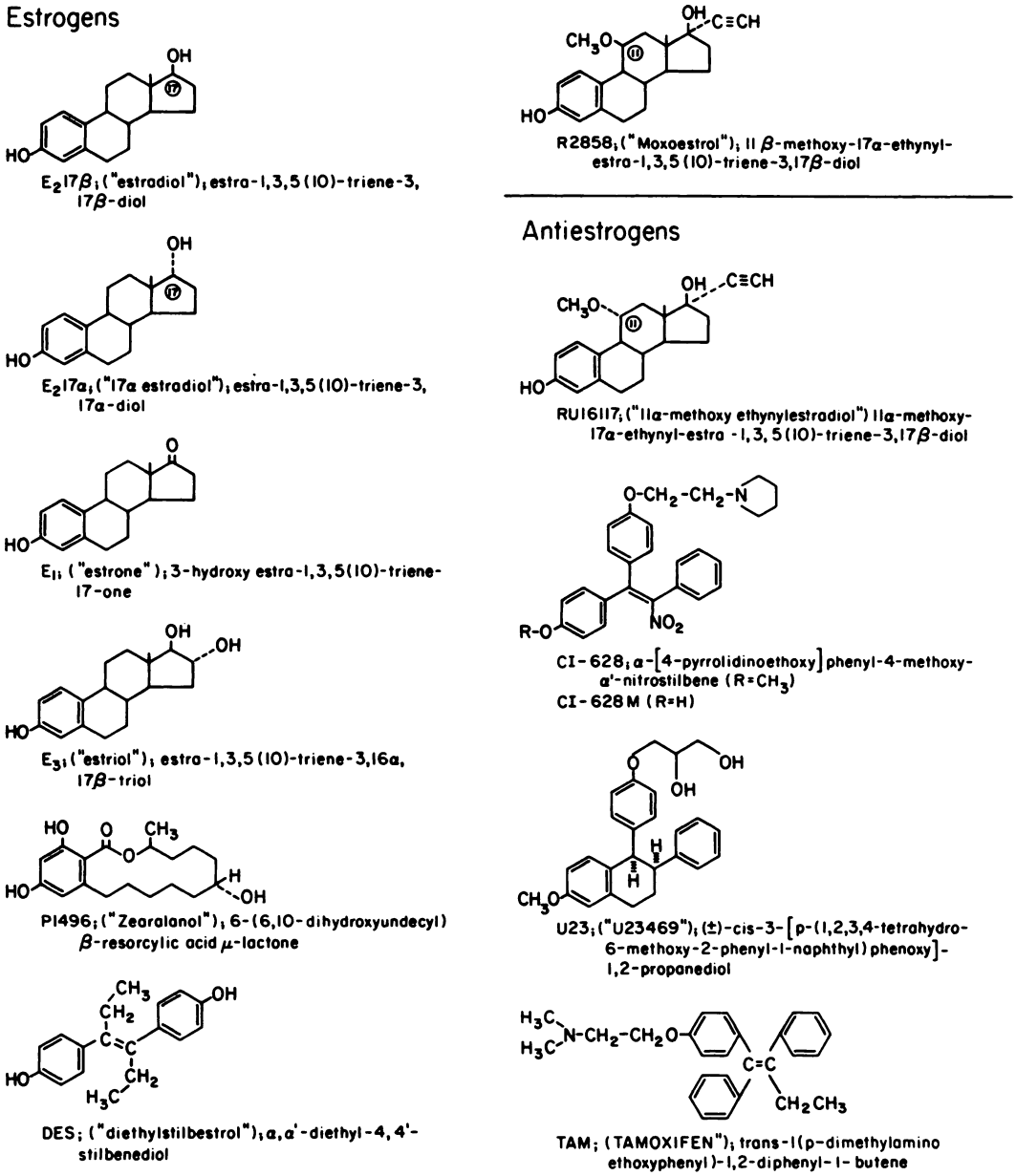

FIG. 1. *Estrogens and antiestrogens: chemical structures and names with abbreviated reference designation*

equivalent to those exhibited when the receptor preparation is fully saturated with estradiol (data not shown).

RESULTS

In the absence of estradiol, the binding activity of the estrogen receptor is lost rather rapidly at 30°C; but when filled with estradiol, the estrogen receptor is stabilized, as shown in Fig. 2 and as noted by others (11, 16, 18). In addition, when complexed with estradiol, the estrogen receptor is resistant to attack by trypsin (Fig. 2).

Other ligands, in addition to estradiol-17β, which are capable of interacting with the estrogen receptor were examined for their ability to confer thermal stability on the receptor. Different ligands promoted thermal stability to varying extents, as shown in Figs. 3A and B, in the order: $E_2 17\beta \sim DES \sim R2858 \sim CI\text{-}628M > P1496 > E_3 \sim E_1 > E_2 17\alpha \sim RU16117 > CI\text{-}628 \sim TAM$.

Mild trypsinization at 0°C has been used previously to deaggregate receptor preparations with little loss of ligand binding (19–25); incubation with higher concentrations of trypsin or at higher temperatures causes more rapid ligand dissociation from estrogen receptor, presumably through a proteolysis which makes the receptor less able to bind ligand (26). We have exposed our preparations to trypsin (30 μg/mg cytosol protein) at 30°C and observed a loss of receptor binding activity (Fig. 4). If the preparations are maintained at 0–4°C, little loss of receptor occurs, as reported previously (22, 25).

Resistance to tryptic proteolysis was found to be conferred in a differential manner by various ligands, as shown in Figs. 4A and B, in an order similar to that observed in the promoting of thermal stability: $E_2 17\beta \sim DES \sim R2858 \sim CI\text{-}628M > P1496 \sim E_1 > RU16117 \sim E_3 \sim E_2 17\alpha \sim CI\text{-}628 \sim TAM$. None of the ligands

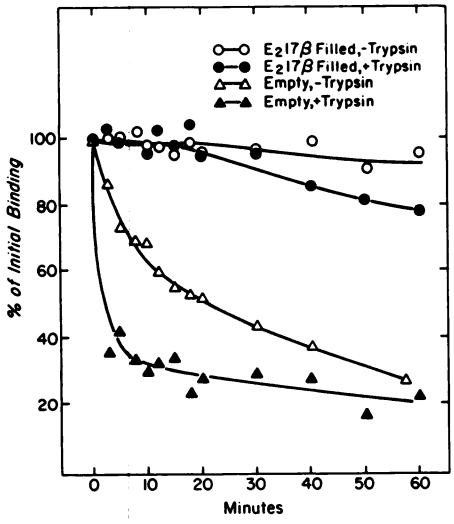

FIG. 2. *The effect of estradiol-17β on the thermal stability and resistance to tryptic proteolysis of the estrogen receptor*

Empty or uncharged receptor was incubated at 30°C in the presence or absence of trypsin (30 μg/mg protein) for periods of up to 60 min. Proteolysis was stopped by cooling and the addition of soybean trypsin inhibitor (2.5 μg/μg trypsin). Radiolabeled estradiol (10 nM) ± 20 μM DES was then added and the receptor charged for 16 h at 0–4°C. For experiments with E_2-17β-filled receptor, freshly prepared cytosol was charged with 10 nM radiolabeled estradiol ± 20 μM DES for 16 h at 0–4°C prior to incubation at 30°C in the presence or absence of trypsin. Results are expressed as the percentage of initial estrogen receptor-specific binding and are the result of duplicate determinations.

interfered with the activity of trypsin as judged by the degradation of [^{14}C]ovalbumin in the presence of each ligand (10 nM; data not shown).

Examination of Figs. 3 and 4 reveals that two processes can describe the phenomena of ligand-mediated thermal stability and ligand-mediated resistance to trypsin. These processes are the initial rate of loss of receptor–ligand complexes and the level to which receptor–ligand complexes ultimately decline. Further analysis of each component process relative to the affinity of different ligands for the estrogen receptor is detailed in Figs. 5A and B.

The relationship between ligand affinity and the initial rate of loss of receptor complexes at 30°C is shown in Fig. 5A as an inverse relationship which tends to a minimum. (Values for the initial rates of loss of receptor complexes were obtained by linear regression analysis of the receptor binding prior to the point at which the constant level was reached.) Compounds which have an affinity at least half as high as $E_2$17β maximally depress the rate of receptor complex loss at 30°C, while compounds which have an affinity less than 20% that of $E_2$17β are able to depress the rate of receptor loss in a manner which appears to approximate a linear inverse function. Log transformation of the rates of loss of receptor–ligand complexes and the relative binding affinity (RBA), shown in the inset to Fig. 5A, demonstrates an inverse linear relationship. A similar analysis applied to the initial rate of loss of receptor–ligand complexes in the presence of trypsin, relative to the binding affinities of the different ligands, shows a similar inverse relationship (Fig. 5B) which becomes more linearized after log–log transformation (inset, Fig. 5B).

Analysis of the relationship of the final constant level of binding relative to ligand affinity for both ligand-mediated thermal stability (Fig. 6A) and ligand-mediated resistance to trypsin (Fig. 6B) also approximates a model where thermal stabilization or protection against trypsin is maximized by compounds having higher affinities for receptor. This final, constant level, and the rate at which it is reached, may be indicative of the process of receptor activation or of receptor heterogeneity (see Discussion).

DISCUSSION

Previous investigations have reported that estradiol-17β protects the estrogen receptor from thermal inactivation (11, 16, 18). A recent report has shown that the rate of thermal inactivation is independent of the protein or receptor concentration and that 5α-dihydrotestosterone can confer some thermal stability while progesterone has no thermal stabilizing capacity (18). Moreover, the fact that the antiestrogen tamoxifen is unable to provide any thermal stabilizing capacity has led to the speculation that antagonists, in contrast to agonists, are unable to induce a conformational change favoring thermal stability in the receptor protein (11). Our data presented in this report extend these observations by evaluating the potential of a variety of estrogen agonists and antagonists at mediating thermal stability and by introducing the phenomenon of ligand-mediated resistance to tryptic pro-

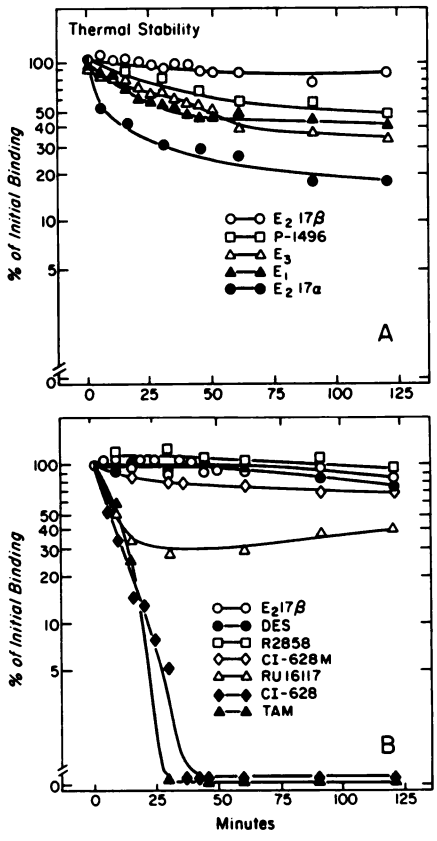

FIG. 3. *The thermal stabilizing capacity of various radiolabeled estrogen and antiestrogen ligands*

Abbreviations for ligands are as defined in Fig. 1. Experiments were performed as described in Materials and Methods and results are expressed as percentages of initial estrogen receptor-specific binding.

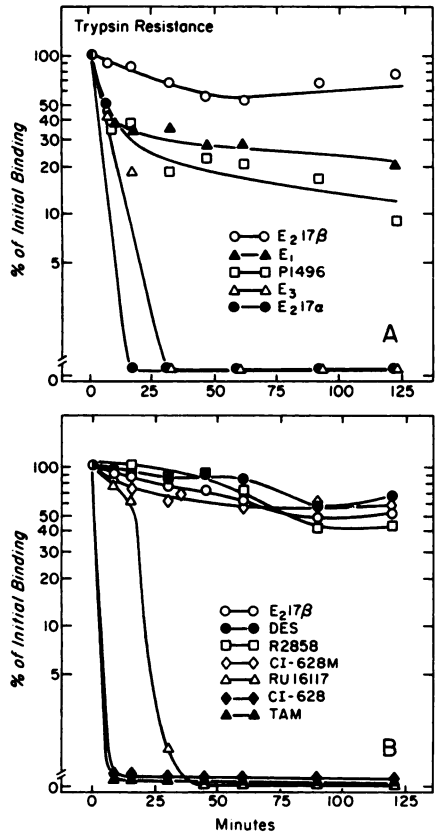

FIG. 4. *The capacity of various radiolabeled estrogen and antiestrogen ligands to promote resistance to tryptic proteolysis*

Abbreviations for ligands are as defined in Fig. 1. Experiments were performed as described in Materials and Methods and results are expressed as percentages of initial estrogen receptor-specific binding.

teolysis. We have found that different ligands vary widely in the degree of thermal stability or protection from tryptic proteolysis that they afford; however, their effectiveness parallels their binding affinity for receptor and not their agonist/antagonist character. Perhaps the most striking demonstration of this is the fact that the high-affinity antiestrogen CI-628M is as effective as estradiol in protecting receptor against thermal- and trypsin-mediated inactivation.

A simple interpretation of these ligand-mediated phenomena is that as a consequence of interaction with ligand, a perturbation of estrogen receptor structure or conformation occurs. As a result of this perturbation, it can be hypothesized that sites on the receptor which are susceptible to attack by trypsin or which are sensitive to thermal denaturation become less accessible. Part of this stabilizing and protective effect of different ligands may be related to their ability to "activate" the estrogen receptor to a more stable form from which ligand dissociates more slowly. This heat activation of receptor (26) could clearly be occurring during the time course of our experiments which were conducted at 30°C.

One hypothesis that would account for the biphasic nature of the loss of receptor binding capacity, that is, the rapid decay followed by approach to a constant level, is that during the course of the binding measurements, receptor is undergoing ligand-mediated thermal activation, whereby it is being converted from an unactivated form that is thermolabile and sensitive to trypsin degradation to an activated form that is resistant to these processes. Recent observations indicating that the rate of receptor activation appears to be related to ligand affinity (26, 27) would be consistent with the observation that the higher-affinity ligands allow more ligand–receptor complexes to survive a 120-min incubation at 30°C. We have not yet evaluated this possibility since such activation studies are complicated by the lability of receptor complexed with low-affinity ligands (28). The biphasic nature of the loss of activity might also reflect a molecular heterogeneity in the receptor population, especially since the addition of fresh trypsin during incubation does not alter the biphasic nature of these phenomena. Alternatively, the biphasic character of the disappearance of receptor binding might be explained by a loss of factors in the cytoplasmic extract influencing receptor stability that occurs during the course of the *in vitro* incubation.

Our motivation in initiating the experiments described here was to explore the possibility that the differences between principally agonistic (estrogens) and principally antagonistic ligands (antiestrogens) might be reflected by differences in the ability of these ligands to induce a

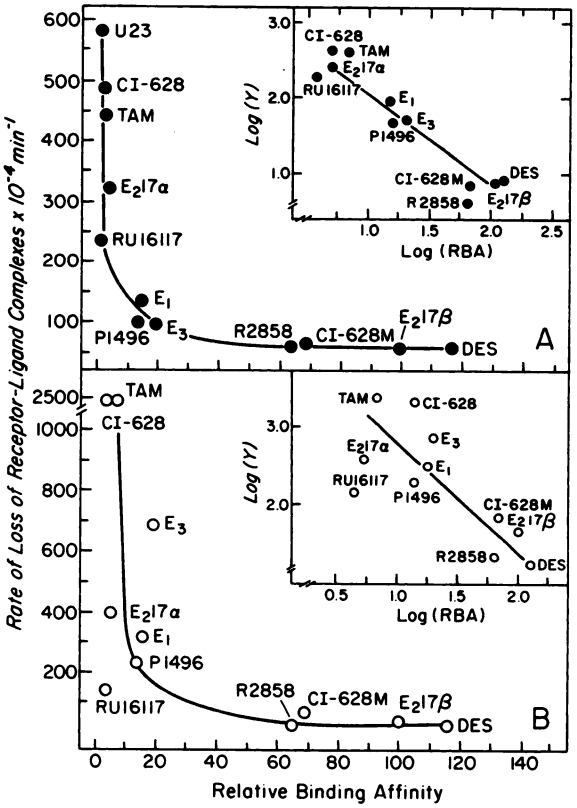

FIG. 5. *Comparison of the ligand relative binding affinity (RBA) to the rate of disappearance of receptor complexes at 30°C (A) and in the presence of trypsin at 30°C (B)*

Experiments and analyses were performed as described in Materials and Methods. The insets express the data after log transformation of abscissa and ordinate values.

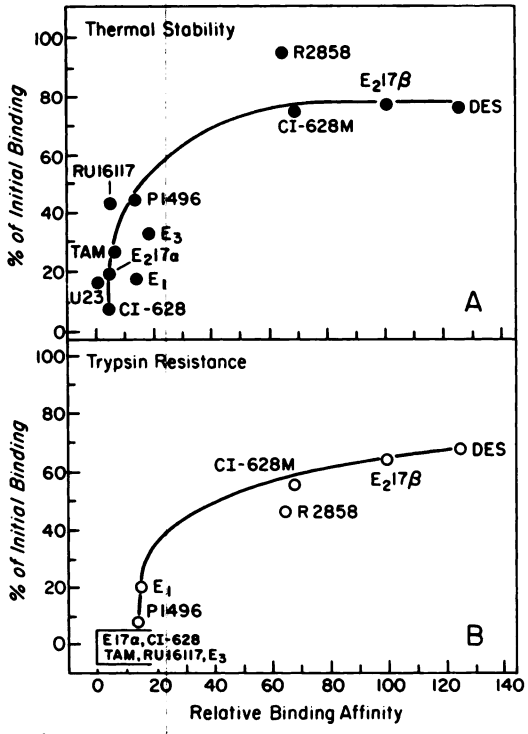

FIG. 6. *Comparison of the ligand relative binding affinity to the level of binding ultimately reached after exposure at 30°C (A) and in the presence of trypsin at 30°C (B)*

Experiments and analyses were performed as described in Materials and Methods.

perturbation which results in thermal stability and/or resistance to tryptic proteolysis. While it is still likely that the estrogen receptor "interprets" an agonist differently from an antagonist, this cannot be translated to a detectable difference between agonists and antagonists in terms of receptor thermal stability or resistance to tryptic attack. Our studies show that both receptor thermal stability and resistance to tryptic proteolysis appear more directly related to a physical property, ligand affinity, than to the biological properties of agonism or antagonism. Because work in this laboratory (13, 29) and elsewhere (30) has shown that high-affinity ligands can function as antiestrogens, it is not possible to discriminate between estrogens and antiestrogens simply by evaluating the binding affinity of a ligand or the thermal stability or resistance to tryptic attack of the cytoplasmic estrogen receptor complexes.

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Send reprint requests to: B. S. Katzenellenbogen, Department of Physiology and Biophysics, 524 Burrill Hall, University of Illinois, Urbana, Ill. 61801.