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Hydrodynamic Characterizations of Estrogen Receptors Complexed with [^{3}H]-4-Hydroxytamoxifen: Evidence in Support of Contrasting Receptor Transitions Mediated by Different Ligands†

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ABSTRACT: Size-exclusion high-performance liquid chromatography was used to characterize the hydrodynamic molecular properties of estrogen receptors complexed with estradiol and the antiestrogen 4-hydroxytamoxifen. Cytoplasmic estrogen receptors complexed with [^{3}H]-4-hydroxytamoxifen did not undergo reductions in hydrodynamic size after exposure to KCl or urea. Nuclear receptors complexed with 4-hydroxytamoxifen eluted as hydrodynamically larger molecules than nuclear receptors complexed with estradiol. Because identical hydrodynamic characterizations were obtained with the covalent ligand [^{3}H]tamoxifen aziridine, these differences in chromatographic behavior are due to differences in ligand-mediated receptor properties and are not the result of ligand dissociation. When estrogen receptors, complexed with either [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen, were exposed to trypsin, the receptors complexed with 4-hydroxytamoxifen eluted as larger hydrodynamic forms than receptors complexed with estradiol. These observations are interpreted to indicate that estradiol and 4-hydroxytamoxifen mediate contrasting transitions in the molecular orientation of estrogen receptors. The consequences of the transitions mediated by 4-hydroxytamoxifen appear to be that intermolecular associations become difficult to disrupt with KCl or urea and that the accessibility of trypsin-sensitive proteolytic sites becomes altered. Chromatin fractionation using DNase I and hypotonic Mg^{2+} solubilization identified a chromatin region that was less readily penetrated by receptors complexed with 4-hydroxytamoxifen than receptors complexed with estradiol. This observation supports the hypothesis that one consequence of different ligand-mediated receptor transitions is that receptors become positioned distinctively in chromatin by agonistic and antagonistic ligands. We suggest that these transitions may be related to mechanisms that separate the actions of estrogen agonists and antagonists.

Estradiol enters the intact mouse uterus and associates with estrogen receptor proteins, which then interact with chromatin (Jensen et al., 1968; Shymala & Gorski, 1969). Some of these interactions appear to take place in a particular chromatin region, which previously has been identified as Mg^{2+}-soluble chromatin through DNase I mediated chromatin fractionation (Scott & Frankel, 1980; Pavlik & Katzenellenbogen, 1982). The action of estradiol can be antagonized by a series of typically nonsteroidal compounds that have a characteristic triphenylethylene structure and that are categorized as "anti-estrogens" (Clark & Peck, 1979; Katzenellenbogen et al., 1979). These antagonists compete with [^{3}H]estradiol for receptor binding sites. Some of these compounds are capable of activating receptors to varying degrees (deBoer et al., 1981; Katzenellenbogen et al., 1981) and of bringing about their retention in chromatin (Clark & Peck, 1979; Katzenellenbogen et al., 1979). Since antagonists ultimately cause receptor sites to be deposited in chromatin, it has so far been difficult to reconcile antagonism through the identification of peculiar or defective receptor–ligand interactions. However, it is well recognized that anti-estrogens promote a more lengthy retention of nuclear receptors than estrogens (Clark & Peck, 1979; Katzenellenbogen et al., 1979). The antagonist 4-hydroxytamoxifen has a high affinity for estrogen receptors (Borgna & Rochefort, 1981) and mimics estradiol with respect to many interactions within the mouse uterus. In this paper we have used SEHPLC[1] analyses to characterize the hydro-

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[1] Abbreviations: CD assay, dextran-coated charcoal assay; DES, diethylstilbestrol (α,α'-diethyl-4,4'-stilbenediol); DME, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; HAP, hydroxylapatite; P_{100} buffer, KH_2PO_4/K_2HPO_4 (100 mM), pH 7.0; SEHPLC, size-exclusion high-performance liquid chromatography; TAM, tamoxifen [1-[4-[2-(dimethylamino)ethoxy]-phenyl]-1,2-diphenylbut-1(*Z*)-ene]; TAMAZ, tamoxifen aziridine [(*Z*)-1-[4-[2-(*N*-aziridinyl)ethoxy]phenyl]-1,2-diphenylbuty-1(*Z*)-ene]; TOT, 4-hydroxytamoxifen [1-[4-[2-(dimethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenylbut-1(*Z*)-ene]; Tris, tris(hydroxymethyl)-aminomethane.

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dynamic behavior of estrogen receptors complexed with estradiol and 4-hydroxytamoxifen in order to identify distinctive molecular properties that might result from interaction with agonists or antagonists.

Experimental Procedures

Materials. Radiolabeled ligands were obtained as follows: (*Z*)-4-hydroxy[*N-methyl*-^{3}H]tamoxifen (76 Ci/mmol), 17β-[2,4,6,7-$^3H_4$]estradiol (104–115 Ci/mmol), and [*ring*-^{3}H]tamoxifen aziridine (25.5 Ci/mmol) from Amersham. Bovine serum albumin, dextran blue 2000, diethylstilbestrol, ferritin, γ-globulin, ovalbumin, trypsin (type XI), trypsin inhibitor (type I-S), sodium thiocyanate, and urea were obtained from Sigma Chemical Co. Other biochemicals were sodium molybdate (reagent grade, Mallinkrodt), Tris (ultrapure grade, Schwarz/Mann, Inc.), and dimethylformamide (Burdick & Jackson Chemicals). Female mice were used between age 20 and age 23 days (CF-1 strain, Harlan Sprague-Dawley, Indianapolis, IN).

Preparative Procedures. Mice were sacrificed by cervical dislocation and decapitation. Uteri were dissected free of fat and mesentery and placed in Dulbecco's modified Eagle medium (DME) made to 1% ovalbumin (w/v), pH 7.4–7.6 maintained with 10 mM Hepes (20–25 uteri in 8 mL). Preparation of cytosols and nuclei and quantitation of the specific binding of [^{3}H]estradiol were as previously described (Pavlik & Katzenellenbogen, 1982; Pavlik et al., 1982a,b, 1985). When [^{3}H]-4-hydroxytamoxifen (10–50 nM) was used, radioinert estradiol (2–10 μM) was employed as the competitor to assess nonspecific binding. Binding of [^{3}H]tamoxifen aziridine (20 nM) to estrogen receptors was determined with excess radioinert estradiol as competitor (4 μM) in the presence of 7.5% DMF as described for [^{3}H]-4-hydroxytamoxifen (Katzenellenbogen et al., 1983). Preparations containing [^{3}H]-4-hydroxytamoxifen and [^{3}H]tamoxifen aziridine were kept shielded from light at all times. The chromatin fractionation procedures have been described previously (Scott & Frankel, 1980; Pavlik & Katzenellenbogen, 1982).

Size-Exclusion High-Performance Liquid Chromatography. All procedures have been described in detail elsewhere (Pavlik et al., 1982a, 1985; Nelson et al., 1984). Buffers were membrane-filtered to remove particles. Receptor preparations were membrane-filtered with low protein binding filters (Millex GV, Millipore Corp.; Pavlik et al., 1985). Isocratic elution was performed with flow-metered pumps (Models 110 and 112, Beckman Instruments). Fractionation over different molecular-size ranges was accomplished on Spherogel exclusion columns (TSK-G2000SW, TSK-G3000SW, and TSK-G4000SW, 7.5 × 600 mm), each of which was fitted with a Guard column (Spherogel-TSK precolumn 2000SW, 7.5 × 100 mm). A syringe-loaded injector (Model 210, Beckman Instruments) was fitted with a 250-μL sample loop. Elution was with P_{100} buffer containing 7.5% DMF. Ultraviolet absorbance was monitored at 280 nm with a fixed-wavelength detector (ChemResearch Model 2020, Isco Co.). Rapid-response fraction collectors (FOXY and CYGNET, Isco Co.) were used to collect samples (1 mL/fraction). The HPLC system was maintained and operated at 2–5 °C in a refrigerated chromatography cabinet (Kelvinator). Column cleaning and maintenance have already been described (Nelson et al., 1984). Elutions of specific binding sites are referenced to the elutions of standard proteins. These referenced elutions are designated by their hydrodynamic property (hdp) and are expressed in terms of the marker proteins eluting before and after any specific binding peak (i.e., hdp = ferritin/bovine serum albumin).

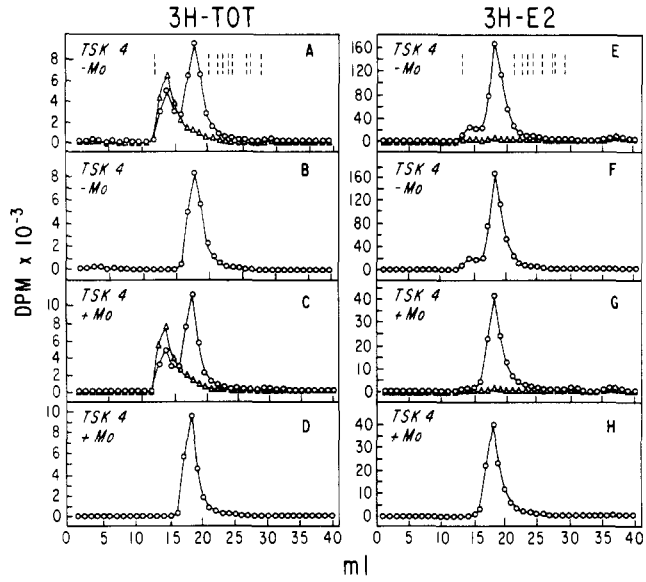

FIGURE 1: SEHPLC analysis on TSK-G4000SW columns of specific binding sites for [^{3}H]estradiol and [^{3}H]-4-hydroxytamoxifen. Cytosols were charged with [^{3}H]-4-hydroxytamoxifen (±2 μM E_2) (A–D) or with [^{3}H]estradiol (±2 μM DES) (E–H). Molybdate was present in preparations C, D, G, and H and absent in A, B, E, and F. Total binding (circles) and nonspecific binding (triangles) are shown in panels A, C, E, and G. Specific binding is shown in panels B, D, F, and H. All determinations were performed on the same cytosolic receptor source. Vertical lines indicate column calibration standard (left to right): dextran blue 2000, ferritin, γ-globulin, bovine serum albumin, ovalbumin, trypsin inhibitor, trypsin, sodium molybdate, and free [^{3}H]estradiol.

Results

SEHPLC Analysis of Estrogen Receptors Complexed to [^{3}H]Estradiol and [^{3}H]-4-Hydroxytamoxifen. SEHPLC analyses were used to characterize cytosolic estrogen receptors complexed with [^{3}H]estradiol and [^{3}H]-4-hydroxytamoxifen (Figure 1). Elution from TSK-G4000SW columns resolved a large nonsaturable binding component only in preparations charged with [^{3}H]-4-hydroxytamoxifen (V_e = 14 mL, Figure 1A,C). Specific binding sites for both [^{3}H]estradiol and [^{3}H]-4-hydrxoytamoxifen eluted later (V_e = 18 mL, hdp = blue dextran/ferritin). In the presence of KCl, cytosolic binding sites for [^{3}H]estradiol were transformed to molecular complexes that had more compact hydrodynamic properties (Figure 2A: hdp = γ-globulin/ovalbumin) than complexes chromatographed in hypotonic P_{100} buffer alone (Figure 1E–H: hdp = blue dextran/ferritin). This observation is consistent with reports that these conditions transform receptors to slower sedimenting forms (Jensen et al., 1968, Notides & Nielsen, 1974, 1975; Notides et al., 1975). Upon being warmed to promote receptor activation, these receptors became less compact hydrodynamically (Figure 2B: hdp = ferritin/γ-globulin) in agreement with reports that increased sedimentation velocity accompanies receptor activation (Notides & Nielsen, 1974, 1975; Notides et al., 1975). NaSCN was not observed to alter the hydrodynamic properties of the cytoplasmic receptor (Figure 2C: hdp = blue dextran/ferritin). Moreover, the addition of urea to the compact KCl-transformed receptor did not result in the receptor becoming hydrodynamically more compact (Figure 2D: hdp = γ-globulin/ovalbumin).

Nuclear estrogen receptors extracted with either KCl or NaSCN eluted with similar hydrodynamic properties on TSK-G2000SW columns (Figure 2E,F: hdp = blue dextran/ferritin). The hydrodynamic properties of nuclear estrogen receptors estimated on TSK-G3000SW columns

Table I: Summary of SEHPLC Elution Analyses of Estrogen Receptors Complexed with Radiolabeled Estradiol, 4-Hydroxytamoxifen, and Tamoxifen Aziridine[a]

		SEHPLC elution of receptor bound to			
	column	[^{3}H]E2	[^{3}H]-4-OH-TAM	[^{3}H]TAMAZ	hydrodynamic size
cytosolic					
hypotonic (a)	TSK-G2000	b. dext/ferr	b. dext/ferr	b. dext/ferr	$E_2(R) = TAM(R)$
hypotonic (a)	TSK-G3000	b. dext/ferr	b. dext/ferr	b. dext/ferr	$E_2(R) = TAM(R)$
hypotonic (a)	TSK-G4000	b. dext/ferr	b. dext/ferr	b. dext/ferr	large nonspecific binding form: TAM(R)
hypertonic, NaSCN (b)	TSK-G2000	b. dext/ferr	b. dext/ferr	b. dext/ferr	$E_2(R) = TAM(R)$
hypertnoic, urea (c)	TSK-G2000	γ-glob/oval	b. dext/ferr	b. dext/ferr	$E_2(R) < TAM(R)$
transformed (d)	TSK-G2000	γ-glob/oval	b. dext/ferr	b. dext/ferr	$E_2(R) < TAM(R)$; only $E_2(R)$ is transformed
activated (e)	TSK-G2000	ferr/γ-glob	b. dext/ferr	b. dext/ferr	$E_2(R) < TAM(R)$; $E_2(R)$ converted to a larger form
trypsin, mild (f)	TSK-G2000	BSA/oval	γ-glob/oval		$E_2(R) < TAM(R)$
trypsin, extended (g)	TSK-G2000	t.i./tryp	BSA/oval		$E_2(R) < TAM(R)$
nuclear					
NaSCN extracted	TSK-G2000	b. dext/ferr	b. dext/ferr		$E_2(R) = TAM(R)$
KCl extracted	TSK-G2000	b. dext/ferr	b. dext/ferr	b. dext/ferr	$E_2(R) = TAM(R)$
KCl extracted	TSK-G3000	BSA/oval	γ-glob/BSA	γ-glob/BSA	$E_2(R) < TAM(R)$
KCl extracted	TSK-G4000	BSA/trypsin	γ-glob/oval	γ-glob/oval	$E_2(R) < TAM(R)$

[a] Data have been summarized from Figures 1–5. Receptor preparations were in P_{100} buffer containing 7.5% DMF (a), in 40 mM Tris–0.5 M NaSCN (b), in 40 mM Tris, 0.4, KCl, 1 mM EDTA, and 3 M urea (c), in 40 mM Tris, 0.4 M KCl, and 1 mM EDTA (d), and in (d) warmed for 45 min at 28 °C (e) and treated with trypsin [12.5 μg mL^{-1} (f) or 166 μg mL^{-1} (g)]. Elution is referenced to peak-height elution relative to protein standards (expressed here as "hdp"). Marker proteins: blue dextran 2000 (b. dext), ferritin (ferr), γ-globulin (γ-glob), bovine serum albumin (BSA), ovalbumin (oval), trypsin inhibitor (t.i.), and trypsin (tryp).

(Figure 2G) have consistently been characterized by a major (hdp = bovine serum albumin/ovalbumin) and minor (hdp = blue dextran/ferritin) elution form. Furthermore, chromatography on TSK-G4000SW columns substantiates the elution of a major (Figure 2H: hdp = bovine serum albumin/trypsin) and minor component (hdp = blue dextran/ferritin). Thus, chromatography of extracted nuclear receptors on TSK-G3000SW and TSK-G4000SW columns provided hydrodynamic characterizations that were consistent with previous reports of nuclear receptor size and sedimentation behavior (Jensen et al., 1968; Shyamala & Gorski, 1969; Harris, 1971).

Cytoplasmic specific binding sites complexed with [^{3}H]-4-hydroxytamoxifen were not transformed by 0.4 M KCl to a smaller hydrodynamic form (Figure 3A) and in contrast to preparations charged with [^{3}H]estradiol could not demonstrate the change to a larger hydrodynamic form related to activation. The hydrodynamic properties of cytosolic receptors complexed with 4-hydroxytamoxifen were not altered by exposure to NaSCN (500 mM, Figure 3B). When buffers containing 400 mM KCl were made to 3 M urea, the hydrodynamic behavior of cytosolic-specific binding sites complexed with [^{3}H]-4-hydroxytamoxifen did not change (Figure 3C), in contrast to receptors complexed with [^{3}H]estradiol. Differences between specific binding sites for [^{3}H]estradiol and [^{3}H]-4-hydroxytamoxifen are tabulated and summarized in Table I.

When translocation was mediated by [^{3}H]-4-hydroxytamoxifen, extracted nuclear receptors appeared to be characterized by quite large hydrodynamic properties when analyzed on TSK-G2000SW columns (hdp = blue dextran/ferritin, Figure 3D,F). These apparent properties have also been observed when extracted nuclear specific binding sites for estradiol were chromatographed on TSK-G2000SW columns [Figure 2E and Pavlik et al. (1985)] but do not occur on TSK-G3000SW columns (Figure 3E). Moreover, in analyses on TSK-G3000SW columns, nuclear-specific binding sites for [^{3}H]-4-hydroxytamoxifen demonstrated a greater hydrodynamic size (hdp = γ-globulin/bovine serum albumin, Figure 3E) than was demonstrated by nuclear-specific binding sites for [^{3}H]estradiol (Figure 2G: hdp = bovine serum albumin/ovalbumin).

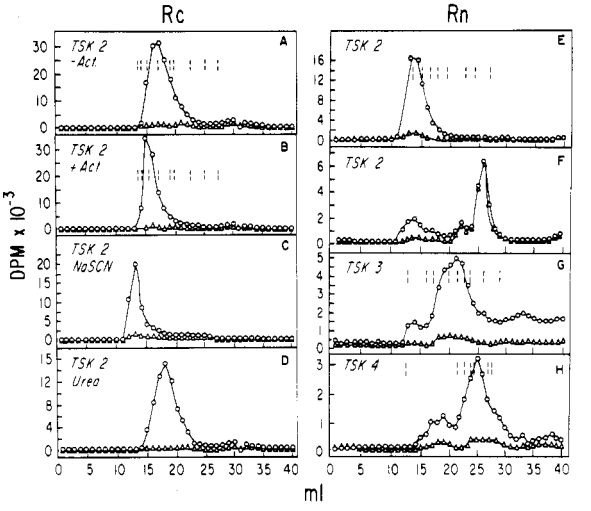

FIGURE 2: SEHPLC analysis of specific binding sites for [^{3}H]estradiol. Cytosolic-specific binding sites (Rc) in high-speed supernatants (five uteri mL^{-1} of P_{100} buffer, 7.5% DMF) were charged with 10 nM [^{3}H]estradiol with and without 2 μM DES as competitor. Total binding (circles) and nonspecific binding (triangles) are plotted in each panel. The columns used were as follows: TSK-G2000SW (A–F), TSK-G3000SW (G), and TSK-G4000SW (H). Vertical dashes indicate the elution of calibration standards (left to right): dextran blue 2000, ferritin, γ-globulin, bovine serum albumin, ovalbumin, trypsin inhibitor, trypsin, sodium molybdate, and free [^{3}H]estradiol. Transformed cytosolic receptor was prepared in P_{100} buffer containing 0.4 M KCl and 40 mM Tris (A). Activated cytosolic receptor was generated by warming transformed receptor (28 °C for 45 min) (B). Elution of cytoplasmic receptors prepared in P_{100} buffer made to 0.5 M NaSCN (C) or made to 3 M urea–0.4 M KCl (D). Receptors were translocated in vitro in the presence of 20 nM [^{3}H]estradiol ± 4 μM DES for 20 min at 37 °C (E–H). Nuclear estrogen receptors (Rn) were extracted with 0.4 M KCl (0–4 °C, 60 min) (E, G, and H) or with 0.5 M NaSCN (F). Preparations containing nuclear-specific binding sites (Rn) were filtered and eluted at 0–4 °C (E–H). Free steroid was removed with dextran-coated charcoal before injection. All preparations were filtered with Millipore GV filters prior to injection. Elution was with P_{100} buffer containing 7.5% DMF at 1 mL min^{-1} (0–4 °C).

The differences observed here between estrogen receptors complexed with [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen might be explained by some dissociation and rebinding of 4-hydroxytamoxifen. As a consequence, analysis of 4-

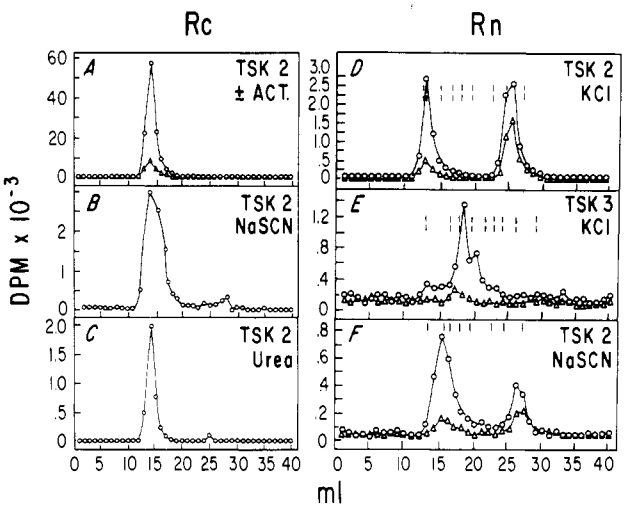

FIGURE 3: SEHPLC analysis of specific binding sites for [^{3}H]-4-hydroxytamoxifen. Preparations were charged with 10 nM [^{3}H]-4-hydroxytamoxifen (± 2 μM E_2). Elution of preparations in P_{100} buffer, P_{100} buffer containing KCl (0.4 M), Tris (20 mM), and P_{100} buffer containing KCl and Tris after warming (28 °C, 45 min) and eluted similarly (A). Cytosolic receptor prepared in P_{100} buffer made to 0.5 M NaSCN (B) or made to 3 M urea (C). Elution was on TSK-G2000SW columns (A–D) as described in Figure 1. In vitro translocation (37 °C, 20 min) was performed in DME–1% ovalbumin containing 20 nM [^{3}H]-4-hydroxytamoxifen (±4 μM E_2). Washed nuclei were extracted either with 400 mM KCl–10 mM Tris, pH 7.4, before injection to TSK-G2000SW (D) and TSK-G3000SW columns (E) or with 500 mM NaSCN–10 mM Tris, pH 7.4, before injection to TSK-G2000SW columns (F). Column calibration standards were as in Figure 1. Specific binding is shown in panels B and C. All designations are as in Figure 1.

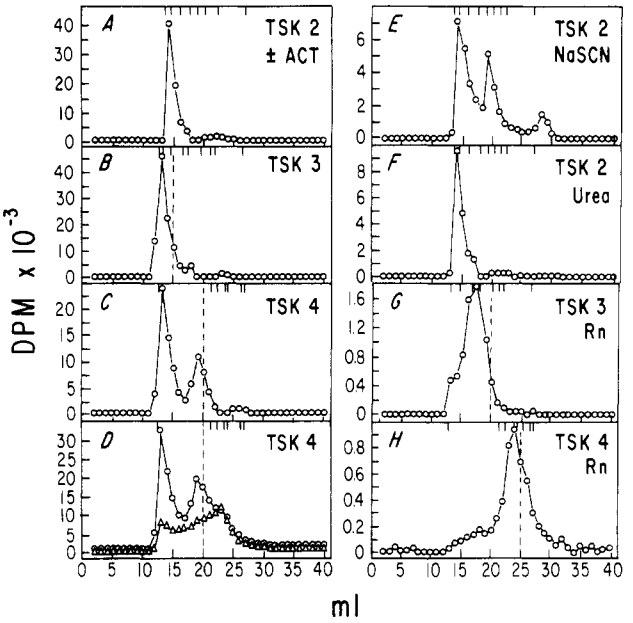

FIGURE 4: SEHPLC analysis of [^{3}H]tamoxifen aziridine interaction with specific binding sites. Preparations were charged with 20 nM [^{3}H]tamoxifen aziridine (±4 μM E_2). Cytosolic receptors (A–F) were run on TSK-G2000SW columns (A and E–F), TSK-G3000SW columns, (B), and TSK-G4000SW columns (C and D). Specific binding is shown, except for (D) where total (circles) and nonspecific binding (triangles) are shown. Cytosolic binding sites in P_{100} buffer with and without KCl (0.4 M) and after warming (28 °C for 45 min in the presence of 400 mM KCl) all eluted similarly (A). Receptors in the presence of 500 mM NaSCN (E) and 3 M urea (F). Nuclear receptors (G and H) were eluted from TSK-G3000SW (G) and TSK-G4000SW columns (H). Column calibration standards are as shown in Figure 1, except that free steroid elution is not included. Vertical dashed lines are reference guides to eluted fractions of 15, 20, or 25 mL.

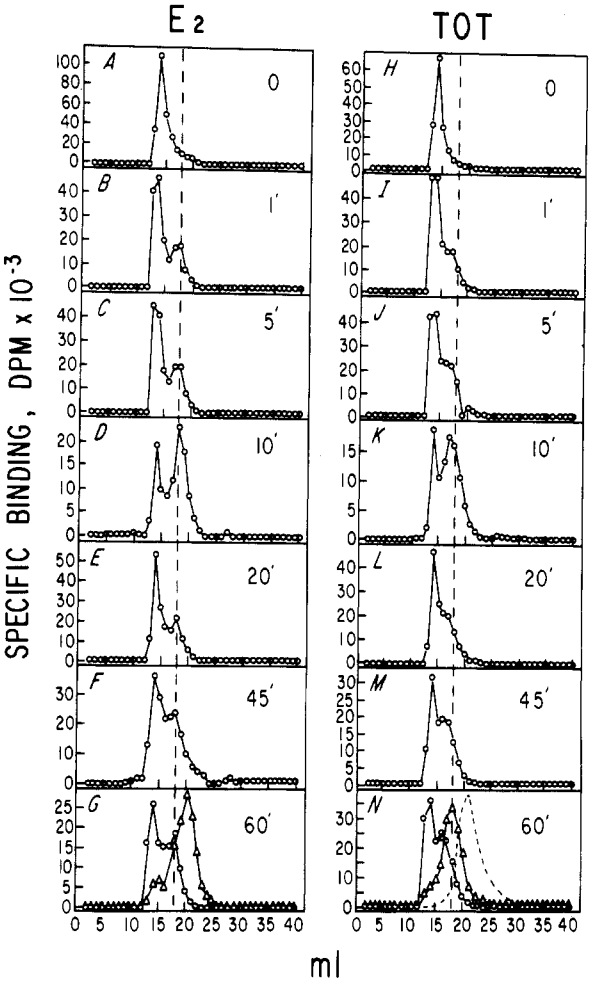

FIGURE 5: Trypsin treatment of estrogen receptors charged with [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen. Preparations were charged with 10 nM [^{3}H]estradiol (±2 μM DES, A–G) or with 10 nM [^{3}H]-4-hydroxytamoxifen (±2 μM E_2, H–N). Matched preparations were then exposed to trypsin (12.5 μg mL^{-1}) for 0–60 min, 0–4 °C. Proteolysis was stopped with soybean trypsin inhibitor (2 μg/μg of trypsin). These procedures have been described previously (Pavlik & Katzenellenbogen, 1980; Pavlik et al., 1982a, 1985). SEHPLC analysis was performed on TSK-G2000SW columns. The vertical dashed lines references the 18-mL fraction, at which point the proteolyzed receptors complexed with [^{3}H]estradiol have peak elution. Elutions after extended exposure to trypsin: 166 μg of trypsin/mL, 60 min, 0–4 °C (triangles, G and N). (Dashed line panel N) Continued exposure to trypsin: 166 μg mL^{-1}, 120–180 min, 0–4 °C.

hydroxytamoxifen receptor complexes that are undergoing rebinding to some extent may represent receptors undergoing a "relaxation" to a receptor state associated with unfilled sites. This possibility was examined by using [^{3}H]tamoxifen aziridine, which forms a covalent linkage to estrogen receptors (Katzenellenbogen et al., 1983). All the characteristics of receptors complexed with [^{3}H]-4-hydrxoytamoxifen were observed when [^{3}H]tamoxifen aziridine was employed (Figure 4 and Table I). These characterizations with tamoxifen aziridine indicate that the elution profiles represent receptor and that the observed differences are not related to ligand dissociation. It is significant that nuclear receptors complexed with the tamoxifen compounds have consistently chromatographed on TSK-G3000SW and TSK-G4000SW columns as larger molecular forms than nuclear receptors complexed with estradiol (Table I).

Trypsinization as a Probe of Ligand-Induced Receptor Conformation. Although proteolyzed estrogen receptors (166 μg of trypsin mL^{-1}, 60 min, 0–4 °C) complexed with [^{3}H]-

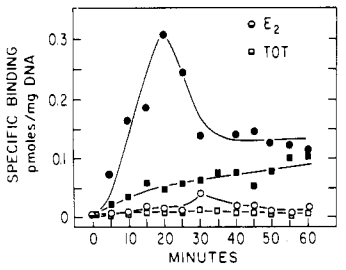

FIGURE 6: Specific binding sites for [^{3}H]estradiol and [^{3}H]-4-hydroxytamoxifen in the Mg^{2+}-soluble chromatin fraction generated by exposure to DNase I. Translocation was allowed to proceed for the times indicated, using 50 nM [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen ± 10 μM competitor (DES or E_2) in DME containing 1% ovalbumin. After translocation with either [^{3}H]estradiol (circles) or [^{3}H]-4-hydroxytamoxifen (squares), partially purified nuclear preparations were subjected to chromatin fractionations with DNase I as previously described (Scott & Frankel, 1980; Pavlik & Katzenellenbogen, 1982). Specific binding activity was determined in the Mg^{2+}-soluble (solid symbols) and Mg^{2+}-insoluble (open symbols) chromatin fractions with the HAP assay (Pavlik & Coulson, 1976; Pavlik et al., 1982a,b). Ten uteri were used in each data point determination. All assay determinations were replicated 4 times. Data are expressed in terms of specific binding. Normalization is based upon total DNA in the preparations determined just prior to nuclease treatment (Burton, 1956). Each nuclease digestion contained 300–700 μg of DNA in 1-mL reaction volumes.

4-hydroxytamoxifen have been observed to chromatograph as larger forms (V_e = 18 mL, hdp = bovine serum albumin/ovalbumin, Figure 5N, triangles) than proteolyzed receptors complexed with [^{3}H]estradiol (V_e = 20 mL, hdp = trypsin inhibitor/trypsin Figure 5G, triangles), continued proteolysis of the 4-hydroxytamoxifen receptor complexes often resulted in a form identical with the trypsinized estradiol receptor complex (dashed profile in Figure 5N). After more limited exposure to trypsin (12.5 μg of trypsin mL^{-1}, 0–4 °C, Figure 5, open circles, using identical cytosols in paired SEHPLC analyses), larger proteolytic products were consistently generated when estrogen receptors were complexed with [^{3}H]-4-hydroxytamoxifen (Figure 5H–N). A straightforward interpretation of these observations is that, as a result of binding either estradiol or 4-hydroxytamoxifen, estrogen receptors undergo different conformational orientations, which result in the exposure of different trypsin-sensitive sites.

A Potential Consequence of Different Receptor Transitions Mediated by Estradiol and 4-Hydroxytamoxifen. Nuclear fractionation studies were performed that utilized exposure to DNase I and hypotonic Mg^{2+} solubilization as previously described (Scott & Frankel, 1980; Pavlik & Katzenellenbogen, 1982) in order to examine the intranuclear distribution of receptors translocated by estradiol and 4-hydroxytamoxifen. The chromatin fraction isolated here (i.e., the Mg^{2+}-soluble chromatin fraction) contains only a portion of the total nuclear receptor activity and consequently provides a good window for comparing the intranuclear distribution of receptors after exposure to either estradiol or 4-hydroxytamoxifen. Receptor activity in the Mg^{2+}-soluble chromatin fraction was much greater when intact uteri were incubated in media containing [^{3}H]estradiol than when the media contained [^{3}H]-4-hydroxytamoxifen (Figure 6). This difference only applied to the Mg^{2+}-soluble chromatin fraction and was not observed in other fractions (i.e., the cytoplasmic, DNase I released, microsomal, KCl-extracted chromatin, or ethanol-extracted chromatin fractions; unpublished results). Because receptors complexed with 4-hydroxytamoxifen penetrate this particular chromatin region with much less facility than receptors complexed with estradiol, each ligand appears to be associated with a somewhat distinctive localization of receptors in chromatin. It is reasonable to hypothesize that this distinctive intranuclear localization results as a consequence of different receptor transitions that are mediated by agonistic or antagonistic ligands.

Discussion

The cogent observations reported here are as follows. First, receptors that are complexed with 4-hydroxytamoxifen, unlike estradiol receptor complexes, are resistant to reduction in hydrodynamic size after exposure to KCl or urea. As a consequence of this resistance, receptors complexed with 4-hydroxytamoxifen have not demonstrated a subsequent increase in hydrodynamic size after being warmed in order to activate receptors. Second, nuclear receptors complexed with 4-hydroxytamoxifen were observed to elute from SEHPLC columns as a larger molecular form than nuclear receptors complexed with estradiol. Third, after exposure to trypsin, receptors complexed with 4-hydrxoxytamoxifen were characterized by greater hydrodynamic size than receptors complexed with estradiol.

Our interpretation of these observations is that estradiol and 4-hydroxytamoxifen may each promote different molecular orientations of the estrogen receptor protein. These distinct molecular orientations would provide a mechanism for explaining the stability of estrogen receptors complexed with 4-hydroxytamoxifen (and tamoxifen aziridine) against deaggregation by KCl and urea. Since extracted nuclear estrogen receptors complexed with the tamoxifen compounds were larger than receptors complexed with estradiol, this stability also has appeared under physiological conditions when receptor binding occurred within the intact uterus. Whether these orientations which result after interaction with the tamoxifen compounds occur as a molecular stabilization against the deaggregation of already formed heteromers or as a status favorable for on-going intermolecular interactions cannot be distinguished at present. The observations that receptors complexed with 4-hydroxytamoxifen exhibited a larger hydrodynamic size after exposure to trypsin than receptors complexed with estradiol also support the concept of contrasting molecular orientations mediated distinctively by estradiol and 4-hydroxytamoxifen. A simple interpretation of this observation is that when receptors interact with each ligand, the orientations associated with ligand binding result in different sets (or numbers) of trypsin-sensitive sites becoming accessible for proteolysis.

A highly relevant association that results from the present chromatographic characterizations is related to the observation that receptors complexed with 4-hydroxytamoxifen penetrated the Mg^{2+}-soluble chromatin much less readily than receptors complexed with estradiol. It seems reasonable to hypothesize that receptor positioning within chromatin is ultimately determined by moleculcar properties that are induced differently by agonistic or antagonistic ligands. For example, *if* chromatin architecture involves microchannel access to the Mg^{2+}-soluble chromatin region, then the same molecular properties of 4-hydroxytamoxifen receptor complexes that limit the probability of hydrodynamic access to chromatographic microchannels might also provide a hydrodynamic limitation to this particular chromatin region. Thus, antagonism could result as a consequence of ligand-mediated transitions, which hinder receptor access to chromatin regions where timely interactions productive to estrogenic responses are initiated.

Other investigators have also reported that estrogen receptors complexed with estradiol and 4-hydroxytamoxifen have different properties. Our observations with mouse preparations are consistent with the earlier reports that the nuclear estrogen

receptor from MCF-7 cells chromatographed and sedimented as a larger form when complexed with 4-hydroxytamoxifen (Eckert & Katzenellenbogen, 1982). MCF-7 receptors, complexed with either estradiol or 4-hydroxytamoxifen, were deaggregated by 3 M urea (Eckert & Katzenellenbogen, 1982) in contrast to the mouse preparations in which only receptors complexed with estradiol were deaggregated. In addition, a polyclonal goat antibody raised against calf nuclear estrogen receptors caused human breast tumor estrogen receptors complexed with 4-hydroxytamoxifen to sediment as a larger form than when estrogen receptors were complexed with estradiol (Tate et al., 1984). Preincubation with this antibody was observed to reduce the specific binding activity of [^{3}H]-estradiol and [^{3}H]DES but *not* of [^{3}H]-4-hydroxytamoxifen (Tate et al., 1984). Finally, chromatography of calf uterine receptor preparations on DEAE-Sephadex has yielded two sharp peaks when receptor was complexed with [^{3}H]estradiol but only one sharp peak when complexed with the antiestrogen [^{3}H]-1285 (Ruh et al., 1983).

In general, we believe that the data reported here and by others strongly support the occurrence of contrasting ligand-mediated molecular transitions that serve to reorient the molecular topology of estrogen receptors. One consequence of the transitional orientations mediated by 4-hydroxytamoxifen appears to be that intermolecular associations become difficult to disrupt (i.e., with KCl or urea). We offer also the simple speculation that these types of reorientation may ultimately limit receptor access to certain chromatin regions (for example, the Mg^{2+}-soluble chromatin) so that the full range of events leading to an estrogenic response cannot be initiated and antagonism results. Although this access limitation results from a straightforward interpretation of our observations and provides a more simplified perception of the mechanisms that separate the action of estrogen agonists and antagonists, additional experimentation will be needed to establish the significance of this concept per se in the elicitation of antagonism.

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