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CHARACTERIZATION OF ESTROGEN AND ANTIESTROGEN BINDING TO THE CYTOSOL AND MICROSOMES OF BREAST TUMORS

M. C. F. Lopes,* M. C. F. Tavares, M. G. P. Vale and A. P. Carvalho
Center for Cell Biology and Hormonology, Faculty of Medicine, University of Coimbra, 3049 Coimbra Codex, Portugal

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Summary—The binding of [^{3}H]estradiol and [^{3}H]hydroxytamoxifen to the cytosol and microsomal fractions of several human breast tumors was investigated. By washing microsomal membranes with a KCl-free or a KCl-containing medium we could distinguish between intrinsic, extrinsic and contaminant estradiol binding sites in these membranes. We observed that treatment of the microsomes with low salt medium removes about 80% of the total estradiol binding sites, whereas 20% are not extractable. The concentration of unextractable [^{3}H]estradiol binding sites in the microsomes varies in proportion to the level of cytosolic estrogen receptors (ER). About 10% of the total extranuclear specific estrogen binding sites was consistently found tightly associated to the microsomal fraction, which displays an affinity for estradiol ($K_d = 0.1$–0.6 nM) similar to that of the cytosolic ER. The displacement of [^{3}H]estradiol with unlabeled hormone or with the antiestrogens, nafoxidine, enclomiphene and tamoxifen (TAM) exhibits identical IC_{50} values either in the cytosol or in the microsomal membranes. On the other hand, the microsomal fraction of breast tumors also binds [^{3}H]hydroxyTAM, but with higher capacity and lower affinity than those of the cytosolic fraction. Furthermore, we did not observe correlation between the concentrations of ER and of antiestrogen binding sites (AEBS) in the tumors. These results indicate that microsomal membranes of human breast tumors contain estrogen binding sites which may be related to the cytosol ER recycling and that specific AEBS are predominantly localized in this membrane system. Furthermore, it is shown that the magnitude of estradiol binding to microsomes depends on the ER positive degree of the tumors, whereas the magnitude of the antiestrogen binding to the microsomes is independent of the ER status of the tumors.

INTRODUCTION

Estrogen receptors (ER) have been found in the nucleus, cytosol and microsomal fractions of several estrogen-target tissues [1–5]. However, the biochemical properties of these receptors and their actual compartmentation is still controversial.

Tamoxifen (TAM), the triphenylethylene antiestrogen currently used in the treatment of human breast cancer, binds to the ER with relatively high affinity ($K_d \approx 80$ nM) [6–8]. It also binds with high affinity ($K_d \approx 1$ nM) and distinctive specificity to another class of intracellular sites, commonly designated antiestrogen-specific binding sites (AEBS) [7–13]. These sites have been found in the cytosol of various tissues [11–14], but recent studies suggest that AEBS are predominantly localized in the microsomal fraction of several estrogen target and non-target tissues [15–20].

The analysis of the ER content in human breast cancer has been useful to evaluate the success of the endocrine therapy with TAM. However, there are some patients whose breast cancers contain high concentrations of ER and do not respond to the drug. On the other hand, some patients with a low level of ER (< 10 fmol ER·mg^{-1} protein) respond to the TAM therapy [7]. It has been suggested that the biological effects of TAM involve the two high affinity systems (ER and AEBS), in addition to low affinity ($K_d \approx 5$–$10\ \mu$M) binding sites, such as calmodulin, protein kinase C, and other cellular receptors which have been proposed as targets for the pharmacological action of TAM [21–26]. A better understanding of the intracellular distribution of these receptor systems and their interrelationship may clarify the

*To whom correspondence should be addressed.

pharmacokinetic mechanism of TAM action as a therapeutic agent of breast cancer.

In vivo, TAM is metabolized to 4-hydroxy-TAM and to *N*-desmethyltamoxifen [27–30]. The hydroxyTAM is an active metabolite whose affinity for the ER is higher than that of TAM [9, 27, 30]. Conversely, the affinity of both antiestrogens for the AEBS is identical [14]. Furthermore, since hydroxyTAM slowly dissociates from ER [7], it appears more stable than TAM and more suitable in estrogen binding studies *in vitro* [9, 14, 25, 31, 32].

In this work, we determined the kinetic parameters for the binding of estradiol and hydroxyTAM to the cytosol and microsomal fractions of human breast cancer specimens. We also investigated whether the microsomal receptors for these ligands are components of the membranes or simple contaminants of the cytosol compartment.

MATERIALS AND METHODS

Chemicals

[2,4,6,7-^{3}H]estradiol (sp. act. 115 Ci/mmol), z-4-hydroxy[*N*-methyl-^{3}H]TAM (sp. act. 85 Ci/mmol), unlabeled 4-hydroxyTAM and unlabeled TAM were purchased from Amersham (Bucks., England); unlabeled estradiol-17β and charcoal Norit A were obtained from Sigma (St Louis, MO); non-radiolabeled antiestrogens (nafoxidine hydrochloride and enclomiphene) were kindly supplied by Upjohn (Kalamazoo, MI) and by Merrel Dow (Cincinnati, OH), respectively.

Tissue sample

Pieces of human mammary tumors (2–5 g) were obtained by surgery performed at the Portuguese Institute of Oncology and at the University Hospital of Coimbra. The tissue was removed and trimmed of excess fat and connective tissue. Shortly after, it was frozen in liquid nitrogen and was stored at −80°C until assayed.

Isolation of cytosol and microsomes from mammary tumors

Frozen tissue was thawed and cut into small pieces in an ice-cold recipient. Homogenization was carried out by using a polytron homogenizer (three 10 s bursts with a 30 s cooling period). To each gram of tissue were added 4–6 ml of homogenization buffer (10 mM TES pH 7.5, 12 mM monothioglycerol, 250 mM sucrose and 20 mM sodium molybdate). The homogenate was centrifuged at 800 *g* for 15 min at 4°C. The resulting supernatant was centrifuged at 10,000 *g* for 10 min, and then it was centrifuged again at 140,000 *g*, for 1 h. The supernatant of the last centrifugation contained the cytosolic fraction and the pellet contained the microsomes.

In some experiments, the microsomes were previously isolated in a salt medium (10 mM TES, pH 7.5, 12 mM monothioglycerol, 250 mM sucrose, 20 mM sodium molybdate and 100 mM KCl) and then, they were washed three times by centrifuging in the same manner with or without 100 mM KCl.

The cytosol and microsomes were immediately utilized in the binding assays or they were stored at −80°C for up to 12 h. All procedures were carried out at 0–4°C.

Quantitative analysis of ER and AEBS binding sites in cytosol and microsomal fractions of mammary tumors

Cytosol or microsomes (150 μl containing 300–400 μg protein) were incubated for 45 min, at 22°C, with 50 μl of radiolabeled ligand ([^{3}H]estradiol or [^{3}H]hydroxyTAM, 0.05–15 nM) in a total volume of 250 μl of a medium containing 10 mM TES pH 7.5, 12 mM monothioglycerol, 250 mM sucrose, 1 mM EDTA and 0.1% bovine serum albumin. Non-specific binding of estrogen or antiestrogen was estimated from parallel incubations containing a 200-fold molar excess of unlabeled estradiol or hydroxyTAM, respectively.

After the incubation period, the reaction was stopped by transferring the tubes to an ice-cold water bath. Thirty minutes later, aliquots of 50 μl of charcoal–dextran suspension (2.5% Norit A and 0.25% dextran T-70) were added to each sample and kept for 10 min with occasional vortexing. Then, the tubes were centrifuged at 3000 *g* for 20 min, at 4°C, and the radioactivity was measured in 200 μl of the supernatant fluid. The specific binding of [^{3}H]estradiol and [^{3}H]hydroxyTAM was subjected to Scatchard analysis [33].

Competition studies between estradiol and hydroxyTAM for the estrogen and AEBSs

The ability of hydroxyTAM to compete with [^{3}H]estradiol for the estradiol binding sites or for the [^{3}H]hydroxyTAM binding sites was determined by incubating cytosol or microsomes (300–400 μg protein) for 45 min, at 22°C, in a

medium containing the radiolabeled ligands [^{3}H]estradiol (1–2 nM) or [^{3}H]hydroxyTAM (3–5 nM) and increasing concentrations of the unlabeled ligands (estradiol or antiestrogens). Bound and unbound ligands were separated by the dextran coated charcoal technique as described above.

Analysis of protein concentration

The protein concentration in the cytosol and microsomal fractions was determined by the biuret method [34]. Before the binding assay, the samples were diluted to 2–3 mg protein/ml with the homogenization buffer.

RESULTS

Characterization of [^{3}H]estradiol binding to the cytosol and microsomal fractions of human breast tumors

The presence of estradiol binding sites in the cytosol and microsomal fractions of mammary tumor cells has been reported by several investigators [4–10, 31]. However, it is controversial whether microsomal receptors are cytosolic contaminants or effective components of the membranes.

We studied the binding of estradiol to breast tumor microsomes isolated in a medium containing 100 mM KCl, and we investigated the effect of membranes washed in a KCl-free or KCl-containing medium. Figure 1 shows the results obtained in one of the three experiments performed with different breast cancer samples. We observed that about 25% of the total estradiol binding sites (B_{max} = 31 fmol·mg^{-1} protein) are removed by simple washing in the isolation medium (KCl present), whereas 80% are removed by washing in a low-salt medium [Fig. 1(A)]. These results indicate that 25% of the total estrogen binding sites found in microsomal membranes are cytosolic contaminants, whereas 20% are receptors which remain tightly associated to the membranes (B_{max} = 7 fmol·mg^{-1} protein) even under conditions of low-salt. The microsomal fraction (55%) which is sensitive to ionic strength changes represents slightly bound ER and it corresponds to the difference between the total removal by low-salt (80%) and the removal which is ionic strength-independent (25%). It appears that all these fractions contain the same type of estrogen binding sites, since they exhibit similar K_d values (0.15–0.3 nM), as determined from Scatchard analysis [Fig. 1(B)].

Figure 2 shows a representative experiment selected from the study performed with ten different ER positive mammary tumors (B_{max} = 20–100 fmol·mg^{-1} protein). The results indicate that the cytosolic fraction contains

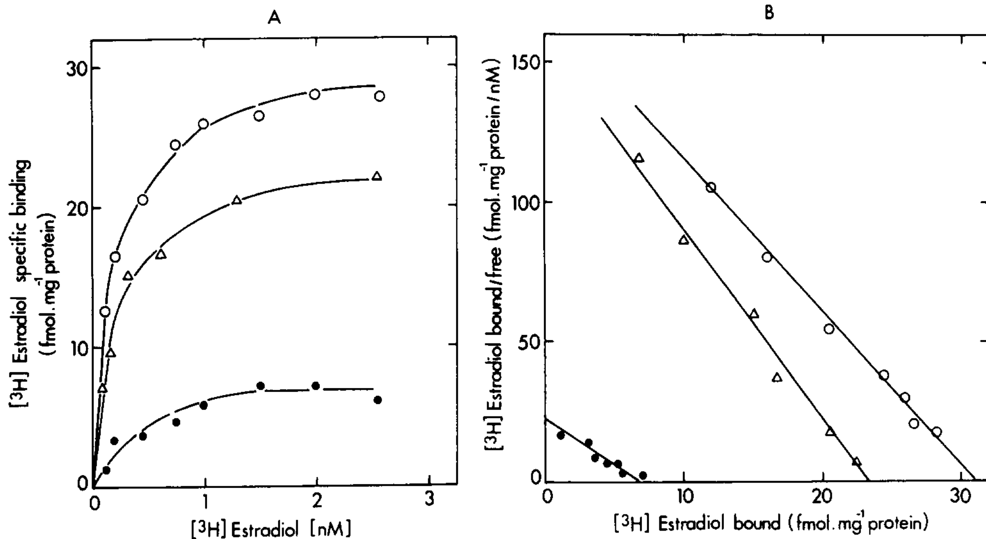

Fig. 1. Differentiation between intrinsic, extrinsic and contaminant estradiol binding sites in breast tumor microsomes. Microsomal membranes were isolated in the presence of 100 mM KCl as described in Materials and Methods. Part of these membranes was washed in the same KCl-containing medium, whereas the other part was washed in that medium, but without KCl. Binding of [^{3}H]estradiol to washed and unwashed microsomes was carried out in the presence and absence of a 200-fold molar excess of unlabeled estradiol. Bound and unbound ligand were separated by dextran coated charcoal technique as described in Materials and Methods. A, saturation curve; B, Scatchard analysis of specific estradiol binding to the membranes. (○) Unwashed microsomes; (△) microsomes washed in KCl-containing medium; (●) microsomes washed in KCl-depleted medium.

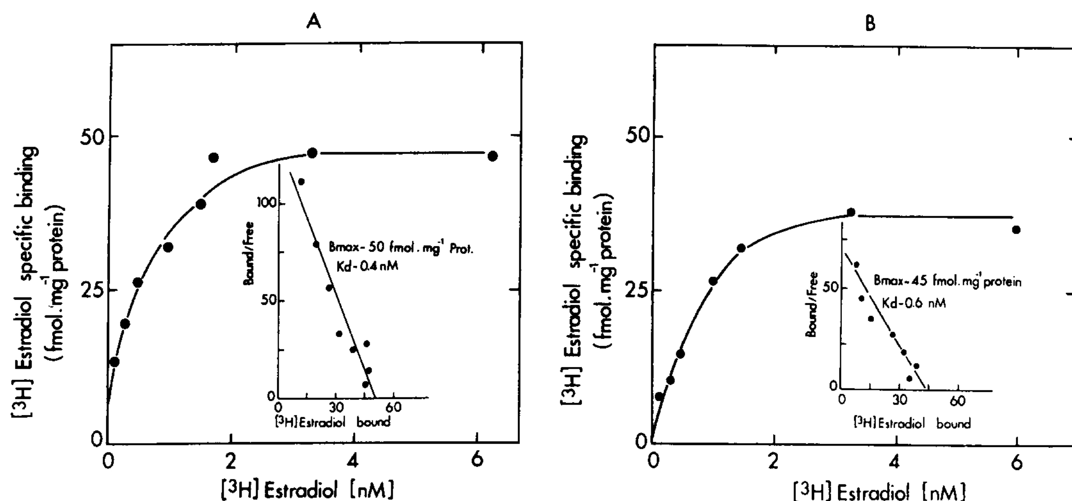

Fig. 2. Binding of [^{3}H]estradiol to cytosol and microsomal fractions isolated from a human breast tumor. The cytosol or microsomes (300–400 μg of protein), isolated in a KCl-free medium, were incubated with 0.05–6 nM [^{3}H]estradiol in the presence and absence of a 200-fold molar excess of unlabeled estradiol as described in Materials and Methods. Bound and unbound ligand were separated by the dextran coated charcoal technique and the specific [^{3}H]estradiol binding was calculated by subtracting the radioactivity measured in the presence of unlabeled estradiol from that measured in its absence. A, specific estradiol binding to cytosol; B, specific estradiol binding to microsomes. Scatchard analysis is plotted insert.

one class of estradiol binding sites with a K_d of 0.4 nM and a B_{max} of 50 fmol·mg^{-1} protein [Fig. 2(A), insert]. Similarly, one type of binding site (K_d = 0.6 nM and B_{max} = 45 fmol·mg^{-1} protein) was detected in low-salt-treated microsomes [Fig. 2(B), insert]. It appears that microsomal membranes contain estradiol binding sites which have binding parameters similar to those of the cytosol.

We also observed that under low-salt conditions (KCl-free medium) the ER distribution between the cytosol and the microsomal membranes was about 82 and 18%, respectively, whereas, in the presence of KCl, the proportional distribution of ER was about 60% in the cytosol and 40% in the microsomes. This indicates that, indeed, microsomes contain slightly associated ER which, in the absence of KCl, appear in the cytosol.

Characterization of [^{3}H]hydroxyTAM binding to the cytosolic and microsomal fractions of human breast tumors

In seven tumor samples, we studied the binding of [^{3}H]hydroxyTAM to the cytosol and microsomal membranes.

Figure 3(A) shows the results of a representative experiment using a tumor with a low level of ER (B_{max} = 10 fmol·mg^{-1} protein), so that its contribution to the antiestrogen binding is negligible. It is evident that the cytosol of the selected tumor contains one class of specific hydroxyTAM binding sites with a K_d value of 0.8 nM and a B_{max} value of 90 fmol·mg^{-1} protein. On the other hand, the microsomes also contain one class of these binding sites but they have higher capacity (B_{max} = 1950 fmol·mg^{-1} protein) and lower affinity (K_d = 3.8 nM) for [^{3}H]hydroxyTAM than those of the cytosolic fraction [Fig. 3(B), insert].

In contrast to estradiol binding, treatment of microsomes with low-salt medium did not significantly alter the binding capacity for hydroxyTAM. After washing the tumor microsomal preparation three times with the low-salt medium, we found a K_d value of 3.2 nM and a B_{max} value of 2150 fmol·mg^{-1} protein [Fig. 3(B), insert], which are similar to those obtained before membrane washing. Below the concentration of 1 nM [^{3}H]hydroxyTAM the binding of the antiestrogen to microsomes appears to be cooperative as revealed by the irregular behaviour observed in the Scatchard plot of Fig. 3(B), insert. Furthermore, we observed that microsomes isolated from different tumors have variable capacity to bind hydroxyTAM (B_{max} values between 120 and 2300 fmol·mg^{-1} protein) (Table 1).

Effect of antiestrogens on the binding of estradiol to cytosol and microsomes of human breast tumors

Competitive studies employing a fixed amount of tracer [^{3}H]estradiol (1.5 nM) and

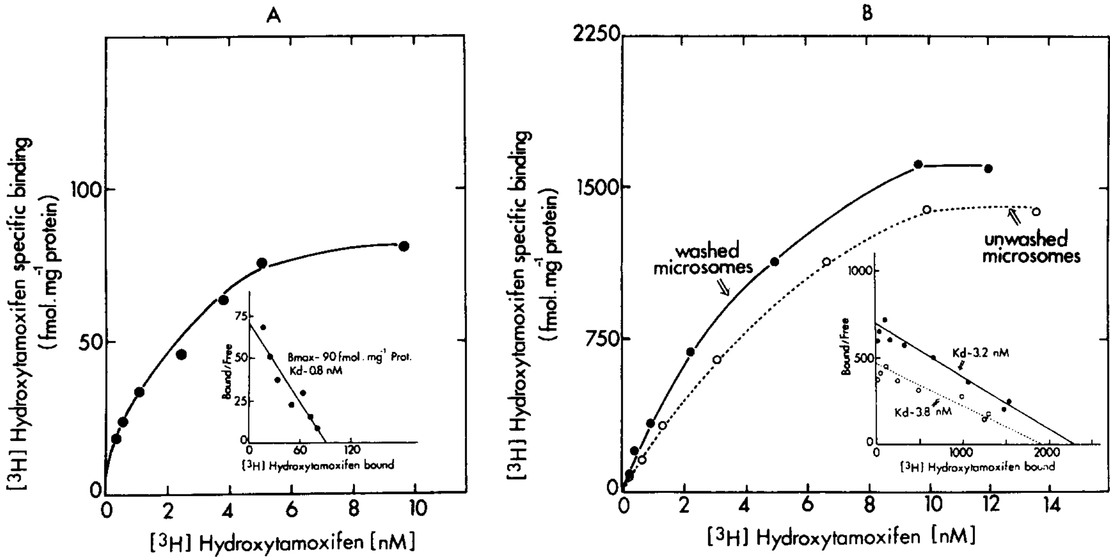

Fig. 3. Binding of [³H]hydroxyTAM to the cytosolic and microsomal fractions isolated from a human breast tumor. Cytosol and microsomes (300–400 μg protein) isolated in a KCl-free medium were incubated with [³H]hydroxyTAM (0.1–15 nM) in the absence or in the presence of a 2-fold molar excess of unlabeled hydroxyTAM as described in Materials and Methods. Microsomes were used in the native state or after washing with the same buffer. Bound and unbound ligand were separated by the dextran coated charcoal technique. Specifically bound [³H]hydroxyTAM was estimated by the difference between the radioactivity obtained in the absence and in the presence of unlabeled hydroxyTAM. A, specific hydroxyTAM binding to cytosol; B, specific hydroxyTAM binding to native and washed microsomes. The (. . .) represents the hydroxyTAM binding to the unwashed microsomes, while the (——) represents the binding to the washed microsomes. Scatchard analysis is plotted insert.

increasing concentrations of unlabeled estradiol or antiestrogens (TAM, nafoxidine and enclomiphene) demonstrated that these compounds inhibited [³H]estradiol binding either in cytosol [Fig. 4(A)] or in microsomes [Fig. 4(B)]. In both fractions, identical IC_{50} values (50–80 nM) were obtained for the antiestrogens, TAM, nafoxidine and enclomiphene. However, when the displacement was performed using hydroxyTAM instead of the other antiestrogens, we observed a lower IC_{50} value, similar to that obtained with estradiol (1–2 nM) [Fig. 4(A) and (B)].

In order to investigate whether the level of ER in tumors influences the estradiol displacement by TAM, we incubated the cytosols from different tumors (B_{max} values between 20 and 200 fmol

Table 1. Determination of the K_d and B_{max} values for [³H]hydroxyTAM binding to the microsomal fraction of different tumors

Microsomes from individual tumors	K_d (nM)	B_{max} (fmol·mg^{-1} protein)
1	3.0	120
2	2.9	200
3	2.0	500
4	5.0	2210
5	3.5	2300

Microsomes isolated from individual tumors were extensively washed in a low-salt medium as described in Materials and Methods. The binding of [³H]hydroxyTAM was performed as described in the legend of Fig. 3.

ER·mg^{-1} protein) with 1.5 nM [³H]estradiol and several concentrations of TAM up to 1 μM. We observed that estradiol specific binding was 50% displaced by 50–80 nM of unlabeled TAM independently of the positive degree of the tumors [Fig. 5 (A) and (B)]. This indicates that the affinity of the estradiol binding sites for TAM is similar either in ER rich tumors or in ER poor tumors.

Competition between estradiol and hydroxyTAM for hydroxyTAM binding sites of cytosol and microsomes of human breast tumors

When increasing concentrations of estradiol were used in competition with [³H]hydroxyTAM, about 40–50% of specific [³H]hydroxyTAM binding to the cytosol fraction of ER positive tumors was displaced with an IC_{50} value of about 20 nM [Fig. 6(A)]. However, the percentage of displacement depends on the number of ER present in the particular tumor studied, which suggests that the cytosolic ER content contributes significantly to the total binding of hydroxyTAM. However, the antiestrogen binding to sites other than ER is not dependent on the ER content of the tumors. In contrast, no significant competitive effect between estradiol and [³H]hydroxyTAM could be measured in

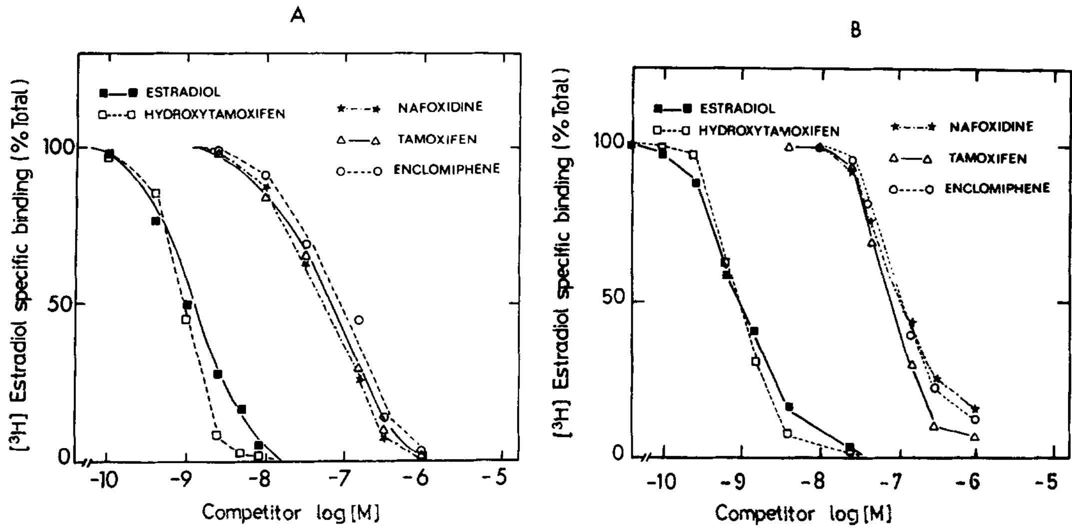

Fig. 4. Competition between [^{3}H]estradiol and various antiestrogens or unlabeled for specific estradiol binding sites of breast tumors. Cytosol or microsomal membranes (300–400 μg of protein), isolated in a KCl-free medium, were incubated with 1.5 nM [^{3}H]estradiol in the absence or in the presence of several concentrations of the non-radioactive competitors (estradiol, hydroxyTAM, TAM, nafoxidine and enclomiphene). Bound [^{3}H]estradiol was determined after incubation for 45 min, at 25°C, as described in Materials and Methods. The estradiol binding obtained in the absence of competitors was set as 100%.

low-salt-treated microsomes of the same tumors, due to the reduced number of ER in these membranes as compared to that of AEBS [Fig. 6(B)].

On the other hand, unlabeled hydroxyTAM (100–1000 nM) promoted 100% inhibition of the [^{3}H]hydroxyTAM binding either in the cytosol or in the microsomal fraction of the tumors [Fig. 6 (A) and (B)]. The IC_{50} values for this competition between labeled and unlabeled hydroxyTAM were slightly lower in the cytosol (30–40 nM) than in the microsomal fraction (60–70 nM) of the tumors.

These results indicate that, in microsomes, the population of AEBS is much higher than that of estrogen binding sites, whereas in the cytosol, the number of AEBS is low and may be equivalent to that of ER when the tumor

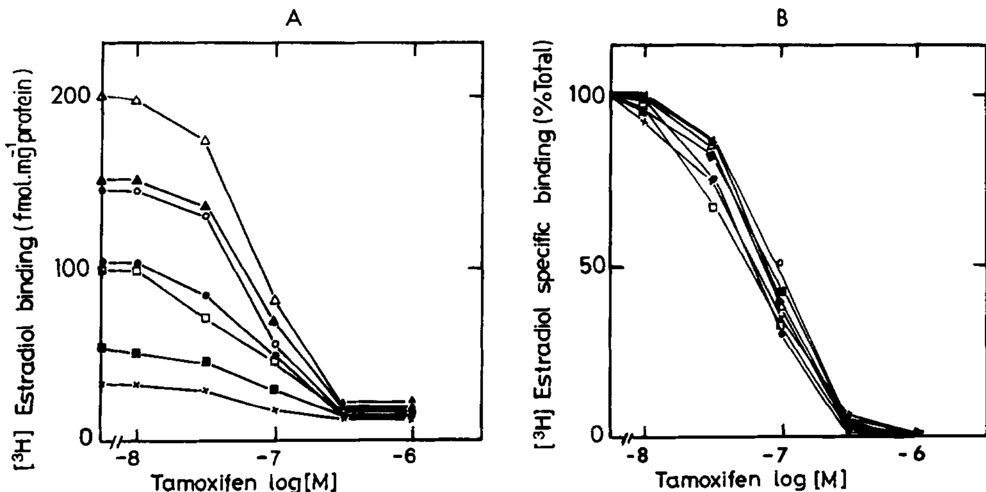

Fig. 5. Effect of TAM on the binding of [^{3}H]estradiol to the cytosolic fraction of human breast tumor biopsies with different ER content. Cytosol (400 μg protein) of several mammary tumors was incubated with 1.5 nM of [^{3}H]estradiol in the absence or in the presence of increasing concentrations of unlabeled TAM up to 1 μM. Bound [^{3}H]estradiol was determined as described in Materials and Methods. A, competitive study as a function of ER concentration ($B_{max} \approx$ 20–200 fmol·mg^{-1} protein); B, results of A expressed as percentage of [^{3}H]estradiol specifically bound to the tumors cytosol. The estradiol binding obtained in the absence of TAM was set as 100%. The various symbols in the figure represent different breast cancer tumor biopsies.

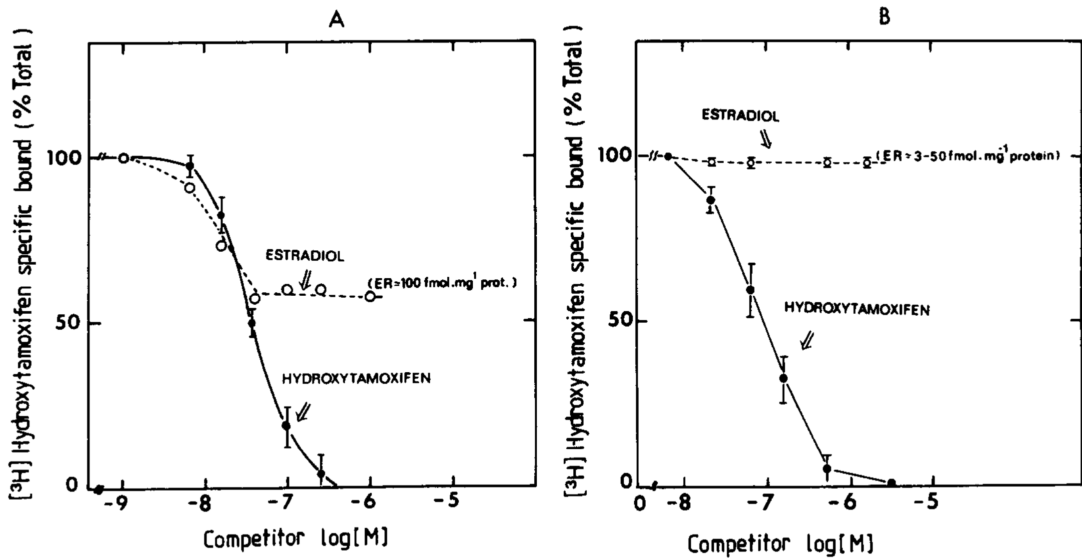

Fig. 6. Competition between [^{3}H]hydroxyTAM and estradiol or hydroxyTAM for [^{3}H]hydroxyTAM binding sites in the cytosol and microsomes of human breast tumors. Cytosol and washed microsomes (300–400 μg protein), isolated in a KCl-free medium, were incubated with 3 nM [^{3}H]hydroxyTAM in the absence or in the presence of increasing concentrations of unlabeled competitors (estradiol and hydroxyTAM). Bound radiolabeled compound was determined as described in Material and Methods. A, binding of [^{3}H]hydroxyTAM to cytosol; B, binding of [^{3}H]hydroxyTAM to microsomes. The arrows indicate the displacers compounds.

is strongly ER positive. In these experiments the number of ER was previously analyzed in the tumors, so that its contribution to the total antiestrogen binding in the cytosol can be evaluated.

DISCUSSION

The biochemical properties of the ER and their localization in different subcellular compartments have been studied by several investigators [1, 5, 35–38]. However, it is still controversial whether the extranuclear population of ER exist only in the cytosol or also in the microsomal fraction.

In this work, we distinguished between intrinsic, extrinsic and contaminant estradiol binding sites in tumor microsomes. These terms were employed to define the binding sites which appear to be tightly associated, slightly associated and not associated to the membranes, respectively. We found that the concentration of estradiol binding sites in microsomes varies proportionally to the level of cytosol ER, and that about 20% of the total membrane binding or 10% of the total extranuclear binding capacity is tightly bound to the microsomes (Figs 1 and 2). Indeed, we found more estrogen binding sites in microsomes of tumor cells whose cytosol is enriched in ER. Since a residual fraction (20%) of the estradiol binding to microsomal sites was not removed from the membranes by treatment with low ionic-salt medium, it appears that they are not cytosol contaminants, but instead, they are microsomal components which are strongly anchored in the membranes. Furthermore, we found a large population (≈55%) of estradiol binding sites, which is removed by low-salt medium but not by KCl-containing medium, suggesting that these sites are extrinsic components of the membranes. On the other hand, only a small fraction of estradiol binding sites found in microsomes (≈25%) represents cytosolic ER contaminants, since they are removed by simple washing of the membranes in a medium similar to that of the microsomal suspension.

We observed that both the intrinsic and extrinsic estradiol binding sites in microsomes have an affinity for estradiol similar to that of the cytosol ER (0.1–0.6 nM) (Figs 1 and 2), and that the displacement of [^{3}H]estradiol with unlabeled hormone or with the antiestrogen, nafoxidine, enclomiphene and TAM exhibits identical IC_{50} values in both fractions (Fig. 4). These results obtained with mammary tumors are in agreement with those of Watson and Muldoon [37] and Parikh *et al.* [38] who demonstrated that there are estradiol binding sites in microsomes of rat and calf uterus which are not simply cytosol contaminants, in spite of their similar properties.

The function of the microsomal estrogen binding sites is not well established although some authors suggest that they modulate the estrogen access to the nucleus or to another extranuclear target system [5]. Since both the intrinsic and the extrinsic microsomal binding sites are not cytosolic contaminants (Fig. 1), it appears that different association states of estradiol binding sites to microsomes may contribute to their overall function in the cells.

Considering that the pool of microsomal ER is essential localized in the endoplasmic reticulum, and that it may be involved in the receptor turnover as a shuttling vehicle for receptor passage into and from the nucleus [5], it is plausible to assume that the microsomal sites represent a form of the receptor which circulates between the nucleus and the cytoplasm. Indeed, at least 40% of the translocated ERs are recycled and reappear in the cytoplasm [2]. The microsomal receptors observed in this work have binding characteristics similar to those of high affinity cytosolic receptors which have been described as the carriers of the hormone message to the nucleus (type I) [2]. Other sites with low affinity (type II) have been reported by other investigators in both the cytosol and the nucleus [39, 40]. They suggested that, in the cytosol, type II sites function in creating an estrogen rich environment for the type I sites, whereas, in the nucleus, they may be involved in the 'processing' mechanism of the type I nuclear complex.

If the functional ER are exclusively localized in the nucleus as it was recently suggested [41], it is plausible to assume that extranuclear binding sites serve to transport the hormone into the nucleus without the cytosolic transforming process preconized by the "two step model".

With respect to the AEBS, we did not observe correlation between their content and that of ER. We found AEBS predominantly in microsomes, although they were also observed in cytosol, in agreement with observations by other investigators [31, 42, 43]. Mehta and DasGupta [44] did not detect AEBS in the cytosol fraction of human breast tumors, probably due to the higher concentration of bovine serum albumin (0.2%) used in their experiments, so that TAM binding to cytosol is masked by the antiestrogen binding to albumin. We also observed that the saturation binding analysis performed in the cytosol either with [^{3}H]hydroxyTAM or with [^{3}H]TAM was identical (data not shown), in agreement with the results obtained by Faye *et al.* [45] using rat uterus cytosol.

In contrast, the IC_{50} values obtained in the displacement of [^{3}H]estradiol with unlabeled hydroxyTAM (Fig. 4) is about 50 times lower than that obtained with unlabeled TAM, which agrees with the higher affinity of the hydroxylated compound for the ER [9, 27, 30]. Comparing the antiestrogen binding to the cytosol and to the microsomal fraction of breast tumors, we found that cytosol binds [^{3}H]hydroxyTAM with higher affinity (5–8 times) and lower capacity (5–25 times) than the microsomal fraction (Fig. 3). These binding properties of cytosol and microsomes indicate that specific AEBS are predominantly localized in the membrane system and that structurally different target molecules for antiestrogens may be operative in both fractions.

The microsomal AEBS were not extracted by treatment with low-salt medium, suggesting that they are intrinsic components of the membranes as it was previously shown by other investigators [17, 18, 20]. They observed that only detergent treatment promotes solubilization of the microsomal AEBS.

In competition studies, we observed that different tumors have similar IC_{50} values for the displacement of [^{3}H]hydroxyTAM by the unlabeled compound [Fig. 6(A) and (B)] which indicates that the same pool of specific binding sites is operating in different tumors.

On the other hand, competition between estradiol and [^{3}H]hydroxyTAM was observed in the cytosol, whereas it was not detected in washed microsomes [Fig. 6(A) and (B)]. This probably reflects that microsomes contain a reduced number of estrogen binding sites, which is masked by the large number of AEBS. Conversely, in the cytosol, the difference between the number of ER and AEBS is smaller, so that estradiol competition is easily observed [Fig. 6(A)].

The nature of AEBS is not known. However, a protein which binds TAM with high affinity has been isolated [12, 43]. Recently, we also showed that TAM binds to calmodulin in a specific, high affinity and Ca^{2+}-dependent manner [26], suggesting that the TAM inhibition of calmodulin-modulated enzymes [46] is mediated by this linkage to calmodulin. Although calmodulin may function as an antiestrogen receptor [7, 21–25], the specific AEBS observed

here appear to be of a different nature, since, in contrast to calmodulin, these AEBS bind TAM in a Ca^{2+}-independent manner. Moreover, as we observed previously [47], calmodulin-independent enzymes may also be sensitive to TAM, suggesting that other molecules besides calmodulin may be targets for the pharmacological effects of the drug.

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