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ESTROGEN-LIKE EFFECTS OF 7,12-DIMETHYLBENZ(a)ANTHRACENE ON THE FEMALE RAT HYPOTHALAMO–PITUITARY AXIS

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Summary—We have recently demonstrated that 7,12-dimethylbenz(a)anthracene (DMBA), a potent inducer of mammary tumors in rodents, can *in vitro* decrease the number of membrane dopamine D_2 receptors and stimulate prolactin (PRL) release, by direct estrogen-like actions on anterior pituitary. In the present study, we tested the ability of DMBA to mimic the *in vivo* estradiol ($17\beta E_2$) effects on pituitary D_2 receptors and on PRL as well as LH release. We have found that DMBA, like $17\beta E_2$, when injected to ovariectomized rats, induced a decrease in the number of anterior pituitary D_2 receptors, a release of PRL and exerted a biphasic (acute negative and longer term positive) action on LH secretion. We thus examined the ability of DMBA to interact with $17\beta E_2$ receptors in the hypothalamo–pituitary axis: DMBA binds to the pituitary cytosolic estrogen receptors with an affinity 0.001% that of $17\beta E_2$. Finally [^{3}H]DMBA binds to hypothalamus-containing brain sections. This binding was displaced partially by RU 2858 a pure estrogen agonist and totally by tamoxifen, a purported estrogen antagonist. No competition for [^{3}H]DMBA binding was observed with an androgen (RU 1881) or a glucocorticoid (RU 26988) agonist.

From these data, it may be concluded that DMBA can act as a partial estrogen in pituitary and hypothalamic tissues.

INTRODUCTION

7,12-Dimethylbenz(a)anthracene (DMBA), a polycyclic aromatic hydrocarbon which is a potent inducer of mammary carcinoma in female rats [1, 2], exhibits structural similarity to estradiol ($17\beta E_2$) [3].

Treatment of Sprague–Dawley female rats with DMBA at 50–55 days of age results in the recurrent enhancement of preovulatory prolactin (PRL) surges [4] as well as PRL surges induced by estradiol benzoate in ovariectomized (ovx) rats [5]. Other groups have provided evidence for a DMBA effect on PRL secretion: Dao and Sinha[6] reported an increase in plasma PRL 6 h after the i.v. injection of DMBA to Sprague–Dawley female rats. Danguy *et al.*[7] showed morphological modifications of anterior pituitary cells which are consistent with an early stimulation of PRL secretion after DMBA administration. Finally, Valero *et al.*[8] showed that like $17\beta E_2$, DMBA treatment rapidly provokes an increase in lactotroph cell numbers, PRL synthesis and glucose-6-phosphodeshydrogenase activity (a $17\beta E_2$-induced enzyme). Recently, we have shown that, like $17\beta E_2$ [9], DMBA *in vitro* exerts a rapid inhibitory effect on the number of AP dopamine (D_2) receptors as well as a stimulatory effect on PRL release [10].

In the present work we investigated the ability of DMBA to mimic the *in vivo* effects of $17\beta E_2$ on pituitary D_2 receptors and on PRL as well as LH release. We also studied the ability of DMBA to compete for [^{3}H] $17\beta E_2$ binding to its pituitary cytosolic receptors. Finally, we used a simple and fast radiochemical assay previously described for glucocorticoid [11] and adapted for estrogen [12] receptor determination in cryostat sections of target tissues, to study [^{3}H]DMBA binding in the hypothalamus.

EXPERIMENTAL

Animals

Female Sprague–Dawley rats (Iffa Credo, Lyon France) weighing 180–200 g were housed under controlled temperature (22°C) and lighting (lights on from 05.00 to 19.00 h), and supplied with water and food (UAR, Versailles, France) *ad libitum*. All rats were bilaterally ovariectomized and used 3 weeks after surgery.

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In vivo effects of DMBA

25 μg of DMBA or 17βE$_2$ in 0.5 ml of sesame oil or the carrier solution alone were injected subcutaneously at 11.00 h (Day 0). Lots of 6 animals from each group were decapitated either on the same day (Day 0) or 2 days after the injection (Day 2), at 16.00, 17.00 and 18.00 h (a period of time at which cycling females exhibit preovulatory PRL and LH surges in the lighting conditions of our laboratory). Trunk blood was collected for serum PRL and LH determinations and pituitary anterior lobes were quickly removed, frozen and stored at −64°C for use within 1 week. The serum was stored at −20°C until PRL and LH assays.

Quantitation of the dopamine D_2 receptors. Preparation of partially purified pituitary membranes, assay for D_2 binding (using [^{3}H]spiperone as the labeled ligand, and *d*-butaclamol in large excess to determine the non specific binding) and determination of protein in the membrane preparations (by the Bradford[13] micro-method) were performed essentially as previously described [14]. A single nearly saturating concentration of [^{3}H]spiperone was used (i.e. 0.784–0.830 nM). This concentration would bind to about 90% of the receptors, assuming an equilibrium dissociation constant (K_d) of 0.090 nM with about 48% specific binding [14]. Higher [^{3}H]spiperone concentrations would occupy more than 90% of the receptors but the percent non specific binding would increase by a greater proportion, thus compromising the ability to accurately estimate the displacable binding. Four total and four non-specific binding determinations were run for each group of pituitaries.

PRL and LH determinations. Sera were analyzed in duplicate by RIA. The values of PRL were expressed as nanogram equivalent of NIADDK rat standard PRL-RP$_3$; the between- and within-assay coefficients of variation were 11 and 7%, respectively. The values of LH are expressed as nanogram of a laboratory rat LH preparation (1.4 × NIH-LH-S$_1$); the between- and within-assay coefficients of variation were 10 and 7%, respectively. Sensitivities of the RIAs for PRL and LH were 0.3 and 0.1 ng/ml, respectively.

Statistical analysis. Differences between group means were evaluated by analysis of variance followed by Student's *t*-tests.

Estradiol receptor assay

Materials. [2,4,6,7-^{3}H(*N*)]-Estradiol ([^{3}H]17βE$_2$; 102 Ci/mmol) was obtained from N.E.N. and unlabeled 17βE$_2$ from Sigma. Tamoxifen (Fig. 1) a purported estrogen antagonist was purchased from Sigma, and DMBA (Fig. 1) from Fluka. Sources of all other chemicals were as follows: Merck (Tris, sodium thioglycolate, sodium molybdate), Prolabo (glycerol), Sigma (dithioerythritol, EDTA, charcoal: Norit-A), Pharmacia (dextran sulfate), N.E.N. (Atomlight).

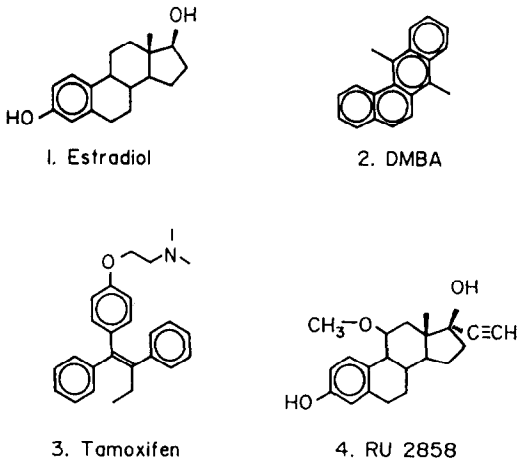

Fig. 1. Structure of 7,12-dimethylbenz(a)anthracene and of some compounds used in this study.

Preparation of cytosolic fractions and receptor assay. Anterior pituitaries were homogenized in an ice-cold 25 mM Tris–HCl buffer (pH 7.4) containing 1.5 mM EDTA, 1 mM sodium thioglycolate, 0.1 mM dithioerythritol, 10 mM sodium molybdate and 10% glycerol. The cytosolic fraction was obtained after centrifugation of the homogenate at 105,000 *g* for 1 h at 4°C. 50-μl aliquots of cytosol were incubated with 0.5 nM [^{3}H]17βE$_2$ (in 50 μl) and increasing concentrations of unlabeled 17βE$_2$, tamoxifen or DMBA, at 4°C for 20 h (A time necessary to achieve binding equilibrium, under our conditions). At the end of the incubation, unbound ^{3}Hligand was removed by addition of 0.25 ml of a suspension of 0.5% charcoal (Norit-A)-0.05% dextran T70 in the homogenization buffer. After centrifugation (20 min at 1800 *g*), 0.25 ml of the supernatants was counted by liquid scintillation in 5 ml scintillation fluid (Atomlight).

[^{3}H]DMBA binding experiments

Materials. [G-^{3}H]7,12-Dimethylbenz(a)anthracene ([^{3}H]DMBA; 50 Ci/mmol) was obtained from Amersham and unlabeled DMBA from Fluka. RU 2858 (Fig. 1; a synthetic estrogen [15]), RU 1881 (a synthetic androgen [16]) and RU 26988 (a synthetic glucocorticoid [17]) were generous gifts from Roussel-Uclaf. Sources of all other chemicals were as follows: Polylabo (Whatman GF/B filter disks). Merck (ammonium molybdate, glycerol, chromium-(III)-potassium sulfate), Sigma (gelatin, ATP, dithiothreitol, EGTA, Tris), Bio-Rad (Coomassie blue) and Baker (Aqualyte).

Preparation of brain sections. The rats were sacrificed by decapitation and the brains rapidly removed and frozen on microtome chucks with dry ice. 32 μm frontal thick sections were cut, using a Bright cryostat at −16°C, at the hypothalamic level (from the optic chiasma to the mamillary bodies: from A 6400 to A 3200 μm according to the stereotaxic atlas of König and Klippel[18]). Brain sections were

collected two by two on glass slides coated with a solution of 1% gelatin and 0.05% chromium(III)-potassium sulfate. The slides were stored at −20°C overnight to allow the sections to adhere to the gelatin coating [19]. The slides were then kept at −80°C until assay.

Incubation of brain sections. Steroids were dissolved in absolute ethanol. [^{3}H]DMBA was kept in toluene which was evaporated before dissolving in ethanol. [^{3}H]DMBA and unlabeled competitors were added to the incubation buffer containing 50 mM Tris–HCl (pH 7.4), 5% (v/v) glycerol, 10 mM dithiothreitol, 2 mM EGTA, 5 mM ATP and 6 mM molybdate. Randomized brain sections were thawed and incubated at 4°C with 300 μl of the buffer for indicated times. Nonspecific binding was measured in the presence of an excess of unlabeled DMBA. After the incubation, the slides were washed 3 times for 5 min each with 200 ml of 50 mM Tris–HCl (pH 7.4) at 4°C. Brain sections were wiped off of the slides with Whatman GF/B filter disks and placed into plastic vials with 5 ml scintillation fluid (Aqualyte) incubated for 15 min at 60°C, cooled and counted in a liquid scintillation counter at 32% efficiency (Packard).

Protein assay. Total protein concentrations were measured on some adjacent sections according to Bradford[13] using bovine serum albumin as the standard.

RESULTS

In vivo estrogen-like effects of DMBA on PRL and LH secretion

When ovariectomized rats were injected with DMBA, or 17βE$_2$ at 11.00 h (Day 0), a significant decrease in the number of available D$_2$ receptors in the anterior pituitary was observed 5, 6 and 7 h later (Fig. 2). Following both treatments, an increase in serum levels of PRL was observed in the first afternoon. However, the DMBA-induced release of PRL was of shorter duration than the 17βE$_2$-induced one.

On Day 2, in DMBA-treated females a small decrease in D$_2$ receptors was observed without any significant change in serum PRL levels. In 17βE$_2$-treated animals a decrease in anterior pituitary D$_2$ receptors and a parallel increase in serum PRL levels were observed at 16.00 h.

DMBA had also clear cut estrogen-like effects on LH secretion: 6 h after treatment, the serum levels of LH were markedly reduced as compared to ovariectomized control rats. The inhibitory effect of both compounds on LH secretion lasted for the 3 studied hours. On the afternoon of Day 2, DMBA-treated rats exhibited an LH surge of an amplitude similar to that observed in 17βE$_2$-treated ones, as a classical result of the positive feedback action of this steroid.

Binding affinity of DMBA for the pituitary estrogen receptor

The binding affinity of DMBA, tamoxifen and estradiol for the cytosolic anterior pituitary estrogen receptors was determined by competitive binding analysis. Tamoxifen and DMBA competed with 0.5 nM [^{3}H] 17βE$_2$ for binding to the estrogen receptor at concentrations above 0.1 and 1 μM, respectively (Fig. 3). Comparison of the concentration needed to produce a 50% decrease in the binding of tritiated 17βE$_2$ ($IC_{50} = 7.5\ 10^{-10}$ M and $4.5\ 10^{-5}$ M, for 17βE$_2$ and DMBA, respectively) indicates that DMBA has an affinity approximately 0.001% that of 17βE$_2$.

[^{3}H]DMBA binding on brain sections

Binding parameters. [^{3}H]DMBA (0.5 nM) was incubated alone (total binding) or in the presence of 500 μM unlabeled DMBA (non specific binding) for indicated times (Fig. 4). The specific binding of [^{3}H]DMBA is expressed as the difference between total and non-specific binding. Preliminary experiments showed that under our experimental conditions, at room temperature the binding was rapid, since half-maximal binding was obtained within 5 min and an equilibrium was reached after 1 h. However non-specific binding represented nearly 50% of the total binding (data not shown). The importance of non-specific binding prompted us to study the kinetic of [^{3}H]DMBA binding at lower temperature. Thus, at 4°C (Fig. 4), an equilibrium was reached after 2 h and half-maximal binding was obtained within 20 min of incubation. In that case, non-specific binding was reduced and represented only about 35% of the total binding. Three hours of incubation at 4°C were then used in subsequent experiments.

Specificity of [^{3}H]DMBA binding. In order to test the specificity of [^{3}H]DMBA binding under these conditions, 0.5 nM of the radiolabeled ligand was incubated for 3 h at 4°C, alone or in the presence of increasing concentrations of various unlabeled competitors. Figure 5 illustrates their relative potency to compete with [^{3}H]DMBA binding in brain sections, expressed as a percentage of initial binding. The order of potency for the [^{3}H]DMBA labeled sites was DMBA > tamoxifen > RU 2858 (a 17βE$_2$ agonist). Whereas tamoxifen and DMBA displaced 80% of [^{3}H]DMBA binding, RU 2858 displaced only 18% of this binding on hypothalamus-containing brain sections. No competition for [^{3}H]DMBA binding was observed with an androgen (RU 1881) or a glucocorticoid (RU 26988) agonist.

DISCUSSION

The present data show that DMBA has significant estrogenic activity not only on anterior pituitary but also on brain tissue. It has been suggested earlier that

this carcinogen, because of its structural similarity to estradiol, is capable of binding to the estrogen receptor [20]. However, the literature concerning DMBA binding to estrogen receptor in uterus and mammary gland remained unconclusive. Though DMBA *in vitro* seems not to compete with $17\beta E_2$ for binding to the estrogen cytosol receptor proteins in rat mammary gland or uterus [21], *in vivo* DMBA treatment increases the number of measurable binding sites for the steroid [21] and its uptake [22] in rat mammary glands. The mechanism of these DMBA actions remained unknown. On the other hand, Lucid and Shaw[23], incubating radiolabeled E_2 and DMBA separately with uterus cytosolic fractions, found that the sedimentation patterns of the two types of complexes formed were identical. The binding of DMBA was inhibited by E_2 and diethylstilbestrol. They concluded that DMBA binds to the estrogen receptor protein. On brain and pituitary, two target organs for $17\beta E_2$ actions [24], no binding data were yet available even though estrogen-like effects of DMBA on anterior pituitary have been reported previously [6–8, 10]. In the present work, we have found that DMBA, when injected to ovariectomized rats can, like $17\beta E_2$, induce a decrease in the number of anterior pituitary D_2 receptors, a stimulation of PRL release and mimic the biphasic (acute negative and longer term positive) feedback action of $17\beta E_2$ on LH secretion. The inhibitory effect of DMBA on D_2 receptor numbers and its stimulatory effect on PRL release, on Day 0,

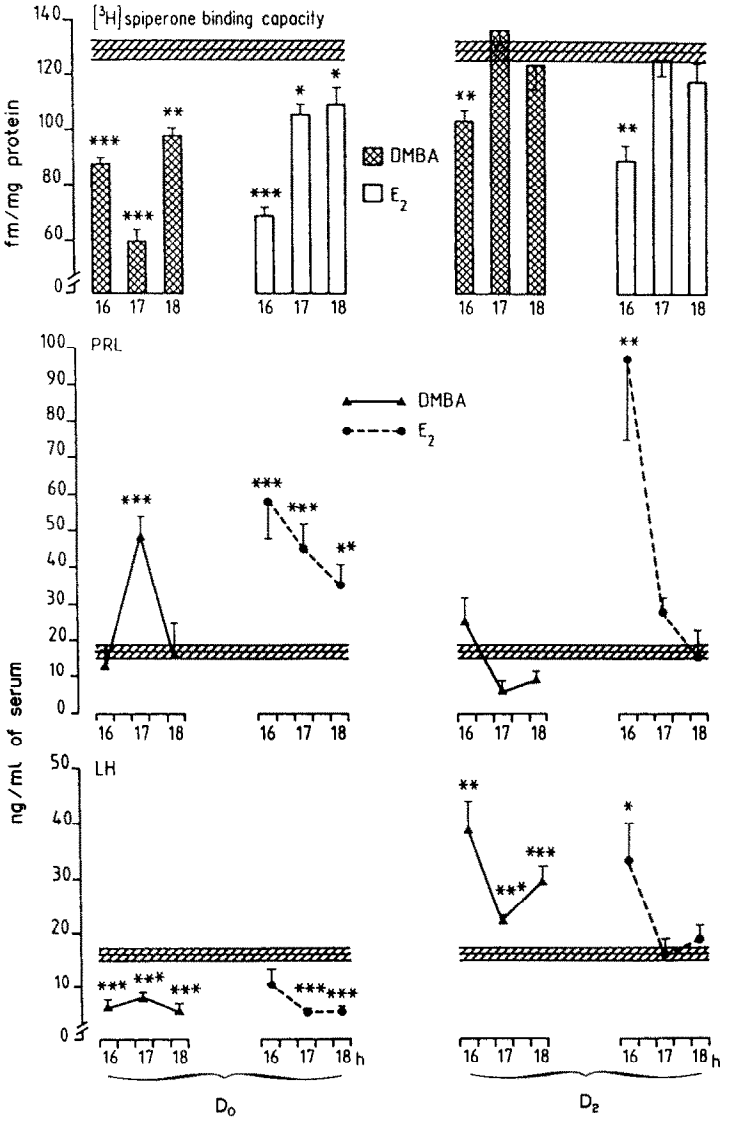

Fig. 2. Effects of DMBA and $17\beta E_2$ on anterior pituitary D_2 receptors ([^{3}H]spiperone binding capacity) and on serum PRL and LH secretions. Three-weeks ovx rats were injected (25 μg s.c.) at 11.00 h and sacrificed at 16.00, 17.00 and 18.00 h, on the day of treatment (Day 0: left panels) and 2 days later (Day 2: right panels). Each binding capacity value is the mean ± SEM of 8 determinations. These values have been compared to the respective control values (means ± SEM: hatched areas) determined at the same time points in vehicle-injected ovx animals (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs controls).

mostly result from a direct action of the carcinogen on the anterior pituitary [10]. Similarly, in order to mimic $17\beta E_2$ negative feedback action on LH secretion [25–28], DMBA may act directly on the pituitary and decrease the sensitivity of gonadotroph cells to GnRH.

To get some insight into the mechanism of these estrogenic actions of DMBA at the pituitary level, we examined its ability to interact with $17\beta E_2$ receptors in the anterior pituitary. DMBA indeed binds to the pituitary estrogen receptors, though the concentration of carcinogen needed to displace 50% of [^{3}H]$17\beta E_2$ binding is high ($\simeq 50\,\mu M$). However, this concentration can accordingly be achieved during several hours in the blood of rats which received 15 mg DMBA by gastric feeding [29], according to the method described by Huggins[1] to induce mammary tumors. In addition, numerous organs (among which the brain) are able to concentrate DMBA, so that local concentrations of the carcinogen in the brain or pituitary might be still more important. Conversely, the 25 μg DMBA we have administered in the present *in vivo* study would not lead to such high concentrations. Yet, estrogenic actions of DMBA can be clearly observed in the absence of $17\beta E_2$. At least two hypothesis can be raised to conciliate the weak potency of DMBA *in vitro*

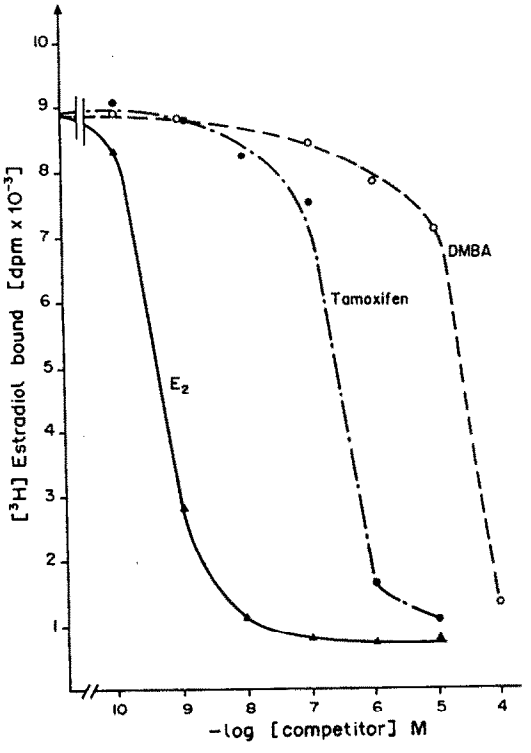

Fig. 3. Competitive binding of DMBA and tamoxifen to the pituitary estrogen receptor. Cytosol from rat anterior pituitaries was incubated for 20 h at 4°C with 0.5 nM [^{3}H]$17\beta E_2$ alone or in the presence of increasing concentrations of unlabeled $17\beta E_2$ (E_2), tamoxifen or DMBA. After adsorption of free [^{3}H]$17\beta E_2$ with charcoal, bound radioactivity was measured. Incubations were performed in duplicate.

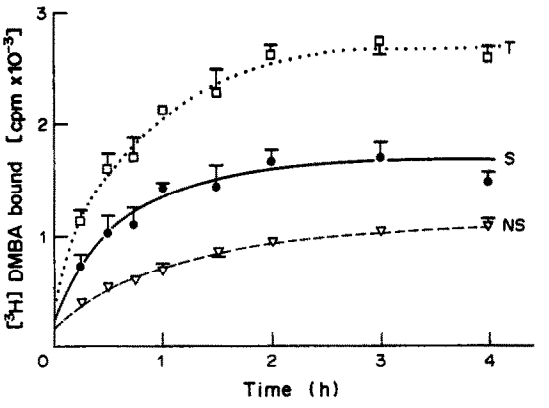

Fig. 4. Kinetic of [^{3}H]DMBA binding. [^{3}H]DMBA (0.5 nM) was incubated alone (total binding; T) or in the presence of 500 μM unlabeled DMBA (non specific binding; NS), at 4°C for indicated times. The specific binding (S) of [^{3}H]DMBA was determined as the difference between T and NS. Each point is the mean ± SEM of four determinations. Three other experiments gave similar results.

to inhibit [^{3}H]E_2 binding and its significant estrogenic potency *in vivo*: (1) DMBA may interact with estradiol receptor at a site distinct from the steroid binding site. (2) *In vivo*, metabolites of DMBA such as its hydroxylated forms are active. Indeed, one of them (3,9-dihydroxyDMBA) has been shown to displace [^{3}H]E_2 binding with an IC_{50} of about 10^{-7} M [30]. Further experiments are needed to clarify that point.

Besides, we also observed in the present work that DMBA induces preovulatory-like LH surges. It is known that in the rat $17\beta E_2$ exerts part of its stimulatory feedback action at the hypothalamic level to trigger a LH surge [31]. Therefore, as confirmed by [^{3}H]DMBA specific binding on hypothalamus-containing brain sections, the hypothalamus could also be a target tissue for DMBA. These data are in agreement with preliminary results from El Abed and Kerdelhué[32] who found high affinity binding sites for [^{3}H]DMBA in the cytosolic fraction of rat hypothalamus. We observed that [^{3}H]DMBA binding was only significantly displaced by RU 2858 (a pure $17\beta E_2$ synthetic agonist) and tamoxifen (a synthetic compound known to interact with estradiol receptor [33–36] and to inhibit the binding of $17\beta E_2$ to its receptor, in the uterus [37] and pituitary [38]). RU 2858 competes for only 18% of [^{3}H]DMBA binding whereas tamoxifen displaces that binding nearly with the same efficacy as unlabeled DMBA itself. It is of interest to draw a parallel between these competition results and those obtained by Furr and Jordan[39] or Sudo *et al.* [40] who studied displacement of [^{3}H]tamoxifen binding by unlabeled $17\beta E_2$ or tamoxifen. Unlike tamoxifen, $17\beta E_2$ can displace only part of [^{3}H]tamoxifen binding. Indeed, it is well known that, besides estradiol receptor, tamoxifen also binds to high affinity saturable binding sites distinct from estradiol receptor [41, 42] which are usually referred to as "antiestrogen binding sites (AEBS)". Thus it might be hypothesized that DMBA also binds to (at

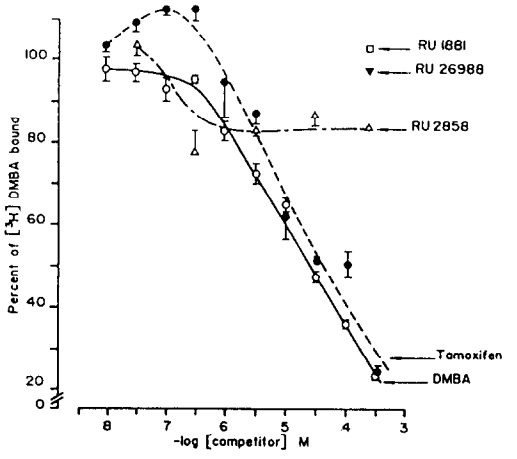

Fig. 5. Specificity of [^{3}H]DMBA binding. [^{3}H]DMBA (0.5 nM) was incubated alone or in the presence of increasing doses of competitors, at 4°C for 3 h. [^{3}H]DMBA binding is expressed as the percentage of the initial binding. Each point is the mean ± SEM of four determinations. Two other experiments gave similar results.

least) 2 different types of proteins: the 18% of [^{3}H]DMBA binding which are displaced by RU 2858 could correspond to estradiol receptors. The remaining 82% of [^{3}H]DMBA binding (displacable by tamoxifen) could correspond to AEBS. These AEBS are uibiquitous proteins [40, 43] and probably not directly involved in mediation of the classically recognized estrogen antagonism of antiestrogens [40, 44]. Actually, tamoxifen is generally accepted to exert many of its biological activities through interactions with estradiol receptor systems [45–48]. Moreover, it is known that in cultured pituitary cells, tamoxifen at low concentrations [49–51] can act as an estrogen agonist. Thus, it is possible that DMBA, either like tamoxifen [52, 53] through binding to estradiol receptor, or indirectly through binding to some other unknown protein which in turn interacts with this receptor, can partially activate this receptor, increase its affinity for some estrogen-responsive elements on DNA and trigger the synthesis of some estrogen-inducible proteins.

We conclude from the present results that DMBA, whatever its real mechanism of action, can act as a partial estrogen agonist on pituitary and also hypothalamic tissues. Since DMBA-induced rat mammary tumor development and growth are well known to be dependent on PRL [54], the fact that DMBA can increase PRL secretion *in vivo* is likely relevant to its carcinogenic effect on mammary gland.

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