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In Vitro Estrogen-like Effects of 7,12-Dimethylbenz(*a*)anthracene on Anterior Pituitary Dopamine Receptors of Rats

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ABSTRACT

The ability of 7,12-dimethylbenz(*a*)anthracene (DMBA), a potent inducer of mammary tumors, to mimic short term effects of estradiol (17β-E_2) on the anterior pituitary, was tested *in vitro*. Incubation of anterior pituitaries from ovariectomized rats with DMBA resulted in a marked depletion of membrane dopamine receptors (labeled with [^{3}H] spiperone) and a parallel stimulation of prolactin (PRL) release. Maximal receptor depletion and PRL release were obtained after 15–30 min of incubation with 10^{-6} M DMBA. These effects were reversible and already significant after a 5-min incubation. Their magnitude, dose dependency, and time course were identical to those reported for 17β-E_2. A structurally related noncarcinogenic polycyclic aromatic hydrocarbon, phenanthrene, had no effect on [^{3}H]spiperone binding or PRL release. When DMBA and 17β-E_2, at suboptimal concentrations, were simultaneously added to the culture medium, no synergistic effect could be observed. When 10^{-8} M of both compounds were introduced simultaneously, the decrease in dopamine receptors and the increase in PRL release were not greater than those observed in the presence of 10^{-6} M of only one compound, indicating that the same mechanism(s) can be involved. These data suggest that DMBA in desensitizing lactotrophs to dopamine and in releasing PRL, by direct estrogen-like actions on anterior pituitary, may provide a hormonal state conducive to tumor development.

INTRODUCTION

DMBA,[2] a polycyclic aromatic hydrocarbon which is a potent inducer of mammary carcinoma in female rats (1, 2), exhibits structural similarity to 17β-E_2 (3).

Treatment of Sprague-Dawley female rats with DMBA at 50–55 days of age results in the persistent enhancement of preovulatory PRL surges (4) as well as PRL surges induced by estradiol benzoate in ovx rats (5). Other groups have also 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 AP cells which are consistent with an early stimulation of PRL secretion, after DMBA administration. Finally, Valero *et al.* (8) showed that like 17β-E_2, DMBA treatment rapidly provokes an increase in lactotroph cell numbers, PRL synthesis, and glucose-6-phosphodehydrogenase activity (a 17β-E_2-induced enzyme).

Recently, we have shown that 17β-E_2 *in vitro* exerts a rapid inhibitory effect on the number of AP dopamine D2 receptors as well as a stimulatory effect on PRL release (9).

In the present study, we investigated the ability of DMBA to mimic the *in vitro* short term effects of 17β-E_2 on pituitary D2 receptors and PRL release.

MATERIALS AND METHODS

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

The rats were sacrificed by decapitation and the anterior lobes of their pituitaries were quickly removed, carefully rinsed, and incubated at room temperature for different times. The incubation medium for ovx control animals was medium 199 (Flobio, Courbevoie, France) supplemented with 0.1% bovine serum albumin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma, St. Louis, MO). Experimentally treated AP were incubated in the same medium containing different concentrations of DMBA (Fluka, Buchs, Switzerland) or 17β-E_2 (Sigma). According to previous results (10), the maximal blood concentration that the carcinogen can reach after a gastric feeding with 15 mg is 10^{-5} M. Thus the highest dose of DMBA tested was 10^{-4} M. Phenanthrene (Prolabo, Paris, France), a chemically related noncarcinogenic hydrocarbon, was also tested. Stock solutions of DMBA, 17β-E_2, or phenanthrene were prepared in ethanol and diluted in the incubation medium; the final ethanol concentration never exceeded 0.001%. The used incubation medium was stored at −20°C until PRL and LH concentrations were determined.

[^{3}H]Spiperone Binding. The preparation of partially purified pituitary membranes, the assay for D2 binding (using [^{3}H]spiperone as the labeled ligand, and *d*-butaclamol in large excess to determine the nonspecific binding) and the determination of protein in the membrane preparations (by the micro-Bradford method) were performed essentially as described previously (11). However, due to the limited availability of the tissues, in some experiments, a single nearly saturating concentration of [^{3}H]spiperone was used (*i.e.*, 0.784–0.83 nM). This concentration would bind to about 90% of the receptors, assuming an equilibrium dissociation constant (K_d) of 0.09 nM with about 48% specific binding. Higher [^{3}H]spiperone concentrations would occupy more than 90% of the receptors but the percentage of nonspecific binding would increase by a greater proportion, thus compromising the ability to accurately estimate the displaceable binding. Three total and three nonspecific binding determinations were run for each group of pituitaries.

PRL and LH Determination. Incubation media were analyzed in duplicate by radioimmunoassays as described previously (9).

Statistical Analysis. Differences between group means were evaluated by analysis of variance followed by *t* tests. All Scatchard analyses of saturation data were fitted by linear regression analysis.

RESULTS

Effect of DMBA on Anterior Pituitary Spiperone Binding Characteristics. The affinity of [^{3}H]spiperone binding to AP membranes, characterized by an equilibrium dissociation constant (K_d) of approximately 0.09 nM, was unchanged by incubation for 30 min with DMBA: the Scatchard analysis (Fig. 1) yielded parallel straight lines with a K_d value of 0.094 ± 0.005 nM (mean ± SEM of six determinations: $r = 0.97 \pm 0.02$). In contrast, [^{3}H]spiperone binding capacity in APs showed a dose-dependent decrease as a function of DMBA concentration in the incubation medium (B_{max}, from 126.5 fmol/mg protein for controls to 82 or 67.8 fmol/mg protein in APs incubated with 10^{-8} or 10^{-6} M DMBA, respectively) (Fig. 1).

Relationship between Decrease in [^{3}H]Spiperone Binding Capacity and Release of PRL Induced by DMBA. The B_{max} in APs from ovx rats decreased in a dose-related fashion when incubated for 30 min with increasing concentrations of DMBA or

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[2] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; 17β-E_2, 17β-estradiol; PRL, prolactin; ovx, ovariectomized; AP, anterior pituitary; LH, luteinizing hormone.

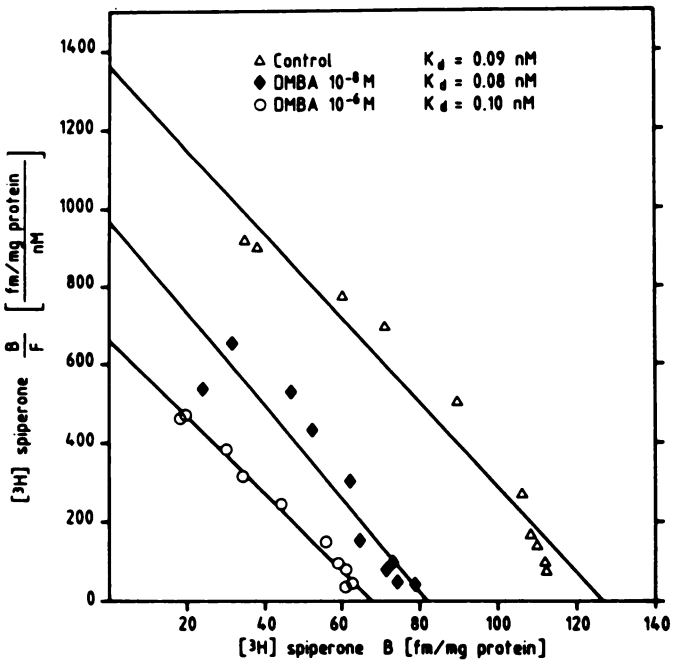

Fig. 1. Scatchard plot of [^{3}H]spiperone-specific binding to AP membranes prepared from ovx rats, when groups of 10 APs are preincubated *in vitro* at room temperature in absence or in presence of DMBA. Each value is the mean of triplicates from a typical experiment. *B*, [^{3}H]spiperone bound; *F*, [^{3}H]spiperone free.

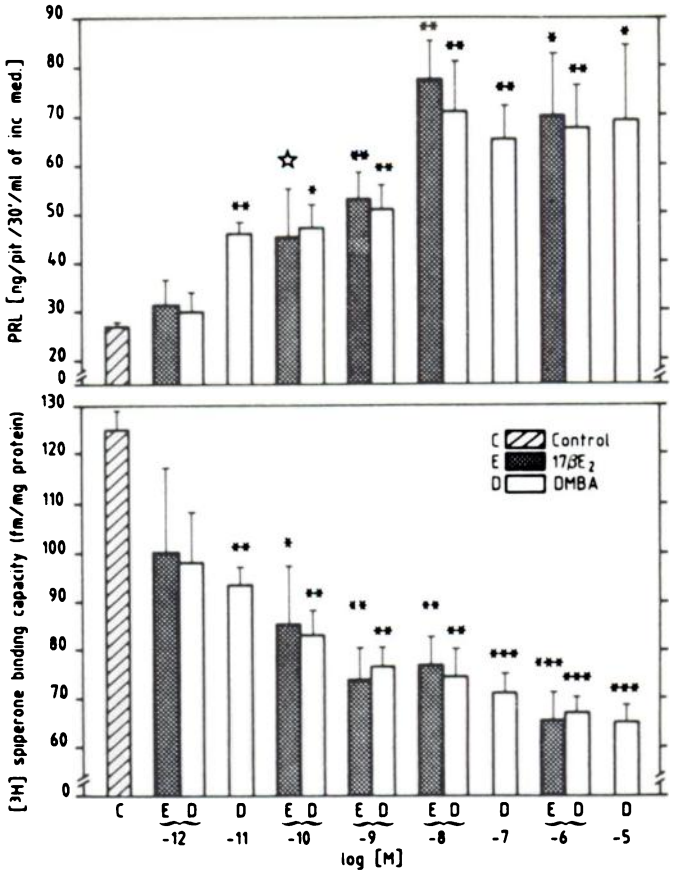

Fig. 2. Relationship between changes in AP D2 receptors (*bottom*) and the release of PRL (*top*). Whole APs were preincubated for 30 min at room temperature, with increasing concentrations of DMBA or 17β-E_2. For binding capacity, means ± SEM of four independent experiments are shown at each concentration. For PRL concentration, means ± SEM are plotted with eight determinations for each concentration (☆, $P < 0.1$;*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus control).

17β-E_2 (Fig. 2). The maximal effect was obtained with 10^{-9}–10^{-8} M DMBA or 17β-E_2, since the decrease obtained with 10^{-7} M to 10^{-4} M (Fig. 2 and Table 1) of either compound was not significantly more important. As with 17β-E_2, variations of [^{3}H] spiperone binding capacity were negatively correlated with the dose-dependent increase in PRL release into the incubation medium. Maximal PRL release was obtained with 10^{-8} M of both compounds. Thus, when both parameters are considered together, maximal effect was obtained with 10^{-8} M of DMBA or 17β-E_2.

Basal LH release was not affected by DMBA treatment (33 ±2.6 ng/AP.30 min and per milliliter of control medium and 33.5 ± 1.3 ng/AP.30 min and per milliliter of medium containing 10^{-8} M DMBA).

Effect of a Structurally Related Noncarcinogenic Polycyclic Aromatic Hydrocarbon. To test if the decrease in [^{3}H]spiperone binding capacity and the release of PRL induced by DMBA had any significance for its carcinogenic potency, phenanthrene was tested in the same *in vitro* system. When added to the incubation medium of APs, phenanthrene had no effect on [^{3}H] spiperone binding (B_{max}, 127.7 ± 5.5 fmol/mg protein for APs incubated in the control medium and 128.6 ± 2.2 fmol/mg protein for APs incubated with 10^{-7} M phenanthrene) or PRL release (data not shown).

Time Course of DMBA Effect on Pituitary [^{3}H]Spiperone Binding and PRL Release. In the presence of 10^{-8} M DMBA, the content of AP D2 receptors declined very rapidly while PRL release increased. These effects were already significant after a 5-min incubation and maximal between 15 and 30 min of incubation (Fig. 3).

Reversibility of DMBA Effects. APs from ovx rats, first incubated for 30 min with 10^{-8} M DMBA, were rinsed and reincubated for 30 min in a control medium. No difference could be found between these APs and APs incubated for 30 or 60 min in a control (DMBA-free) medium, as regards the number of D2 receptors or the amount of PRL released.

Interaction between DMBA and 17β-E_2 Effects (Table 1). When suboptimal doses ($<10^{-8}$ M) of each compound were added simultaneously, additive effects were observed on [^{3}H] spiperone binding (B_{max}, 81 ± 2 or 83 ± 4 fmol/mg protein for APs incubated, respectively, with 5. 10^{-9} M 17β-E_2 or DMBA alone was significantly higher, $P < 0.05$ and 0.01, respectively, than that determined when both were present at this concentration: 71 ± 2 fmol/mg protein) and on PRL release (data not shown). When both compounds were simultaneously introduced at the concentration of 10^{-8} M, the decrease in AP D2 receptors and the stimulation of PRL release were never greater than those obtained in the presence of 10^{-8} M of only one compound. Neither synergistic or antagonistic interaction was observed when a fixed dose of one compound was used in the presence of increasing doses of the other.

DISCUSSION

The present results show that *in vitro* DMBA, like 17β-E_2 (9), can rapidly decrease the number of D2 receptors in APs from ovx rats without modifying their affinity for [^{3}H]spiperone. This dose-dependent DMBA-induced decrease in D2 receptor content is also negatively correlated with an increase in the release of PRL. The dose dependency and the time course of these effects are identical for DMBA and 17β-E_2 (9). Produced either by DMBA or 17β-E_2, these effects are rapidly reversible. No synergism or antagonism between DMBA and

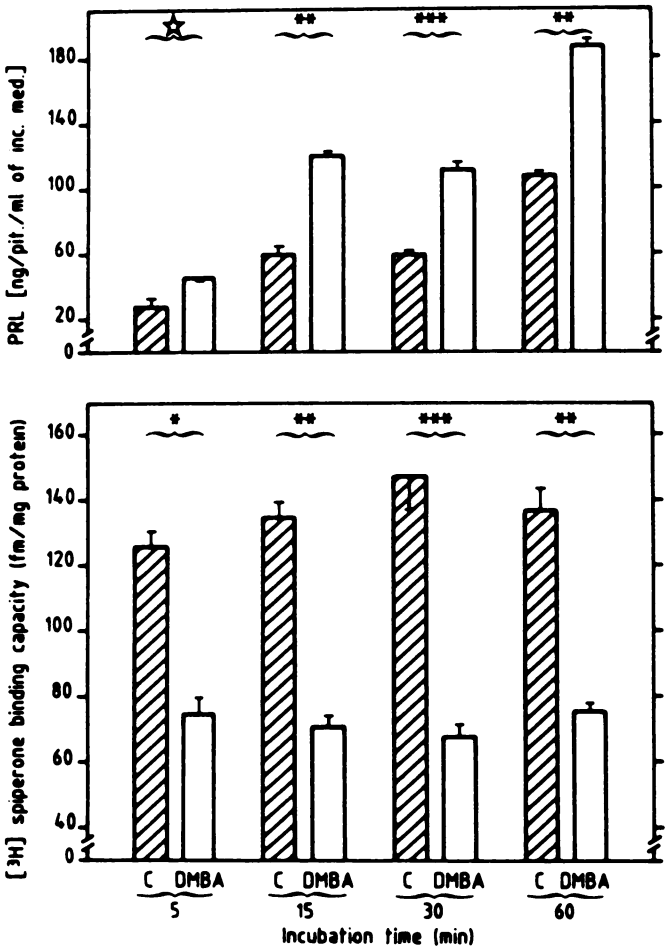

Fig. 3. Time course of the decrease in AP D2 receptors (*bottom*) and the increase in PRL release (*top*), in the presence of 10^{-8} M DMBA. B_{max} values are the means ± SEM of two to five independent experiments. For PRL concentration, means ± SEM are plotted with four to 10 determinations for each time point (☆, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ versus control).

17β-E_2 can be observed. Their effects on [^{3}H]spiperone binding and PRL release are additive only up to a final concentration of 10^{-8} M. This is consistent with the idea that the same mechanism(s) can be involved in these actions of 17β-E_2 and DMBA. Thus, *in vitro*, DMBA can perfectly mimic two specific biological actions of 17β-E_2 (9) and be considered here as an estrogenomimetic agent. These effects of DMBA might be of significance for its carcinogenic potency since a structurally related noncarcinogenic polycyclic hydrocarbon, phenanthrene, has no action on [^{3}H]spiperone binding capacity or PRL release.

A previous work (12) reported that DMBA *in vitro* inhibits PRL release from rat pituitaries. This discrepancy is likely to be due to the difference in time and volume of incubation: In Ingleton's work, the glands are incubated for 5.5 h in a small volume (five times smaller than in our studies). It is possible that the final reduction of PRL secretion as compared to control pituitaries results from the combination of two phenomena: (*a*) an early stimulation of PRL release (during the first hour) and (*b*) subsequent suppression of its release induced by the high levels of PRL accumulated in the medium, which are known *in vitro* to activate the ultrashort-loop autoregulatory mechanism of PRL secretion (13).

Estrogenic effects of DMBA on AP have been shown previously (8) and it has previously been suggested that the carcinogen, because of its structural similarity to estradiol is capable of binding to the estrogen receptor (14). DMBA indeed interferes with 17β-E_2 binding: although DMBA *in vitro* seems not to compete with 17β-E_2 for binding to the cytosol receptor proteins in rat mammary gland or uterus (15), *in vivo* DMBA treatment increases the number of measurable binding sites for the steroid (15) and its uptake (16) in rat mammary glands. The mechanism of these DMBA actions remains unknown. On the other hand, Lucid and Shaw (17) 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. Preliminary results obtained with brain and pituitary in our laboratory confirmed that part of DMBA binding sites are estrogen receptors.

In any case, the short term estrogenic effects of DMBA described in the present paper are unlikely to be the result of an interaction between the carcinogen and the well-characterized nuclear estrogen receptor. Indeed these effects occur within minutes and are reversible. The presence of specific 17β-E_2 binding sites has been demonstrated on plasma membranes in the uterus (18), liver (19), and brain (20). If specific membrane estradiol recognition sites are also present in the rat AP as recently evidenced by Bression *et al.* (21) it is likely that they mistake DMBA for 17 β-E_2 (the shape of the carcinogen being helpful to this process).

In the absence of DA, the E_2-induced stimulation of PRL release *in vitro* is unlikely to be the result of the decrease in AP D2 receptors. In addition to alter membrane receptors availability to ligands (22), 17β-E_2 also elicits within minutes discharges of calcium action potentials in GH3 cells, which are accompanied by an increase in calcium influx and, hence, a stimulation of PRL release (23).

Whatever the mechanism of action, since DMBA-induced rat mammary tumor development and growth are well known to be dependent on PRL (see Reference 24 for review) the fact that DMBA can directly and rapidly desensitize the pituitary lactotrophs to dopamine and also trigger PRL release might contribute to its carcinogenicity.

These results suggest that DMBA, in addition to initiating neoplastic changes at the mammary gland level, can also, by

Table 1 *Interaction between DMBA and 17β-E_2 for the decrease of [^{3}H]spiperone binding capacity*

B_{max} values (in fmol/mg protein) are the mean ± SEM of three independent experiments.

	17β-E_2						
DMBA	0	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-6} M	10^{-4} M
0	123.9 ± 7.4	95 ± 8.5	85 ± 12[a]	73 ± 7[b]	78.4 ± 5.4[b]	63 ± 7[b]	64 ± 5[b]
10^{-11} M	93 ± 4[a]	94 ± 3[a]			80.25 ± 9.6[a]		
10^{-10} M	84 ± 4.5[b]		80 ± 2.4[b]		79 ± 1.4[b]		
10^{-9} M	76 ± 4[b]			76.3 ± 2.7[b]			
10^{-8} M	74.7 ± 6[b]	78.5 ± 2.7	75.8 ± 3.6[b]		72 ± 3.5[b]	65 ± 3.2[b]	67 ± 7.7[b]
10^{-6} M	65.4 ± 3.9[b]		65.8 ± 4[b]		61.3 ± 8.7[b]		
10^{-4} M	65.8 ± 4[b]				60 ± 5[b]		

[a] $P < 0.05$; [b] $P < 0.01$, Student's *t* test *vs.* control.

direct estrogen-like actions on AP, create a hormonal environment which increases the sensitivity of the mammary gland to tumorigenesis.

Studies to determine if DMBA can mimic estrogen actions at the brain level are currently under way.

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