A paradoxical inhibitory effect of oestradiol-17β on GnRH self-priming in pituitaries from tamoxifen-treated rats

José E Sánchez-Criado, Carmina Bellido, Rafaela Aguilar and **José C Garrido-Gracia**

Department of Cell Biology, Physiology and Immunology, University of Córdoba, Córdoba, Spain

(Requests for offprints should be addressed to J E Sánchez-Criado, Section of Physiology, Faculty of Medicine, Avda. Menendez Pidal s/n, 14004 Córdoba, Spain; Email: fi1sacrj@uco.es)

Abstract

Two-week ovariectomized (OVX) rats were injected over three days with 25 μg oestradiol benzoate (EB), 3 mg tamoxifen (TX) and 0·2 ml oil and their pituitaries were harvested for incubation experiments. Pituitaries from EB- and TX-treated OVX rats exhibited GnRH self-priming when incubated with their corresponding ligand. However, incubation of pituitaries with different ligands yielded divergent results: when pituitaries from EB-treated rats were incubated with 10^{-7} M TX they displayed GnRH self-priming, whereas incubation of pituitaries from TX-treated rats with 10^{-8} M oestradiol-17β (E_2) blocked GnRH self-priming. Further studies to analyse the latter finding revealed that: (a) E_2 inhibited TX-induced GnRH self-priming in a dose-dependent manner while 10^{-8} M oestradiol-17α did not; (b) co-incubation of E_2 with the pure anti-oestrogen ICI 182,780, but not with the selective oestrogen receptor modulator TX, reversed the E_2 inhibitory effect; (c) the oestrogen receptor (ER)-α selective agonist propylpyrazole triol, but not the ERβ selective agonist diarylpropionitrile, mimicked the inhibitory effect of E_2; (d) the analogue membrane-impermeable conjugated E_2-BSA also inhibited TX-induced GnRH self-priming; and (e) a 15-min exposure of the pituitaries to E_2 was sufficient to inhibit the GnRH self-priming elicited by TX. Although other explanations may exist, altogether these results suggested that E_2, via an ER different from classical ER, inhibits the GnRH self-priming elicited by TX.

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Introduction

The self-priming effect of gonadotrophin hormone-releasing hormone (GnRH) is a property of GnRH that increases the magnitude of the luteinizing hormone (LH) response to successive GnRH challenges (Aiyer *et al.* 1976, Fink 1988, 1995, de Koning *et al.* 2001). This property is different from its direct releasing action and is dependent on *de-novo* synthesis of RNA and priming proteins (de Koning *et al.* 1976, Pickering & Fink 1976, Mobbs *et al.* 1990). Experimentally, GnRH self-priming is considered to be present when the magnitude of the LH response to the second (primed response) of two exposures to GnRH separated by an interval of 60 min is significantly greater than the response (unprimed response) to the first exposure of the pituitary to GnRH (de Koning *et al.* 1976). Progesterone receptor (PR) plays a key role in augmenting gonadotroph responsiveness to GnRH (Waring & Turgeon 1992, Turgeon & Waring 1994) and requires previous exposure of the pituitary to oestrogen (Fink 1988) or tamoxifen (Sánchez-Criado *et al.* 2004, 2005).

The triphenylethylene tamoxifen (TX) is a selective oestrogen receptor (ER) modulator that displays mixed agonist/antagonist activities (Cosman & Lindsay 1999, McDonnell 1999, McDonnell *et al.* 2002). In the rat, TX induces *in vivo* and *in vitro* GnRH self-priming in the absence of oestrogen without affecting basal or GnRH-stimulated LH secretion (González *et al.* 2000, Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003). Thus, incubation of pituitaries from TX-treated ovariectomized (OVX) rats with TX produced GnRH self-priming, as did pituitaries from oestradiol benzoate (EB)-treated OVX rats after incubation with oestradiol-17β (E_2) (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). However, the oestrogenic effect of TX on GnRH self-priming disappeared when E_2 instead of TX was added to the incubation medium (Bellido *et al.* 2005). This paper describes this paradoxical inhibitory effect of E_2 and presents evidence that the steroid-specific E_2 inhibition of TX-induced GnRH self-priming probably occurs via an ER different from the classical ER in the gonadotroph.

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Materials and Methods

Animals and surgery

Adult female Wistar rats weighing 200 ± 15 g were used. Rats were housed under a 14 h light:10 h darkness cycle (lights on at 0500 h) at room temperature (22 ± 2 °C) with rat chow and tap water available *ad libitum*. All rats were ovariectomized (OVX) under ether anaesthesia at random stages of the oestrous cycle and were included in the experiments two weeks later. All experimental protocols were approved by the Ethical Committee of the University of Córdoba, and experiments were performed in accordance with the rules of laboratory animal care and international law on animal experimentation.

Treatments

In the first experiment, three groups of OVX rats were injected s.c. over three days with 0·2 ml oil, 25 μg oestradiol benzoate (EB; Sigma Chemical Co., St Louis, MO, USA) or 3 mg tamoxifen (TX; Sigma Chemical Co.). In the second and third experiments only rats injected with 3 mg TX were used. At 0900 h on the first day after treatment, rats were decapitated, the neural lobe discarded and anterior pituitary glands were dissected out and incubated.

General incubation procedure

Incubation of pituitaries was carried out as previously described (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). Briefly, after 60 min preincubation, halves of anterior pituitaries were incubated for 120 min at 37 °C with constant shaking (60 cycles/min) in an atmosphere of 95% O_2-5% CO_2. Each incubation tube contained 1 ml Dulbecco's modified Eagle's medium (DMEM), without L-glutamine and phenol red, containing glucose (4·5 g/l) and bovine serum albumin (BSA, 0·1%, w/v), pH 7·4. Luteinizing hormone-releasing hormone (LHRH; 10^{-8} M) (Peninsula Laboratories Inc., St Helens, Merseyside, UK) was added to the incubation medium for 15 min at the beginning of the first (priming) and second (primed) hours of incubation. All medium was aspirated every 15 min for quantification of LH concentration, and replaced with fresh medium. The last 15-min collection during the preincubation period was used to determine basal LH secretion.

Incubation experiments

In the first experiment, three groups of 12 OVX rats each, treated with oil, EB or TX were used. The 24 hemipituitaries from each treatment group were randomly assigned to three different incubation treatments: medium alone, 10^{-8} M oestradiol-17β (E_2; Sigma Chemical Co.) or 10^{-7} M TX (Sigma Chemical Co.). In the second experiment, hemipituitaries from 84 OVX TX-treated rats were randomly assigned to different incubation treatments as follows: (a) 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} M E_2 or 10^{-8} M oestradiol-17α (Sigma Chemical Co.); (b) 10^{-7} M of the pure antiestrogen ICI 182,780 (Tocris Cookson Ltd, Avon, Avonmouth, UK) or 10^{-7} M TX with or without 10^{-8} M E_2; and (c) 10^{-10}, 10^{-8}, 10^{-6} M of the ERα selective agonist propylpyrazole triol (PPT) (Tocris) (Stauffer *et al.* 2000) or the ERβ selective agonist diarylpropionitrile (DPN) (Tocris) (Meyers *et al.* 2001). Finally, in the third experiment, hemipituitaries from 36 OVX TX-treated rats were randomly assigned to the following different treatments: 10^{-10}, 10^{-8}, 10^{-6} M of the cell impermeant E_2-BSA (Sigma Chemical Co.) and 10^{-8} M of E_2 for 15, 30, 45 or 60 min during the preincubation period immediately before the priming hour. Controls for experiments 2 and 3 included pituitaries from TX-treated OVX rats incubated with medium alone and with TX.

RIA of LH

Concentrations of LH in incubation media were measured in duplicate by RIA using a double-antibody method with kits supplied by NIH (Bethesda, MD, USA) and a previously described microassay method (Bellido *et al.* 2003). Rat LH-I-9 was labelled with ^{125}I by the Chloramine T method. Intra-assay and interassay coefficients of variation were 8% and 9% respectively. Assay sensitivitiy was 3·75 pg/tube. LH was expressed as ng/ml medium of the reference preparation LH-rat-RP-3.

GnRH self-priming

Under the present incubation protocol, the peak pituitary response occurs after 15 min exposure to GnRH challenge (Sánchez-Criado *et al.* 2002, 2004). With the exception of pituitaries from the first experiment (oil-, EB- and TX-treated OVX rats), the peak LH response to the first LHRH pulse in pituitaries (TX-treated OVX rats) was not significantly altered by the test substances added to the medium. Thus, GnRH self-priming was calculated as peak response to the second LHRH pulse × 100/peak response to the first LHRH pulse (de Koning *et al.* 1976, Sánchez-Criado *et al.* 2005); 100% or less indicated absence of GnRH self-priming.

Statistical analysis

Statistical analysis was performed by ANOVA to test the existence of significant differences among groups. When significant differences existed, it was followed by the Student-Newman-Keuls multiple range test for intergroup comparison. Significance was considered at the 0·05 level.

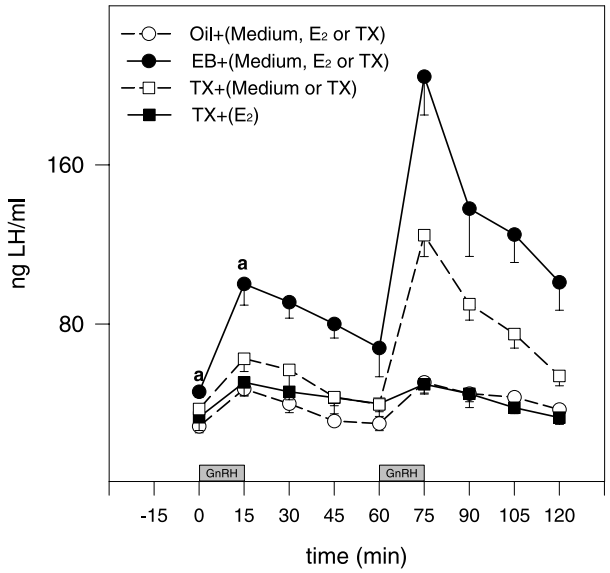

Figure 1 LH secretion from two-week OVX rat pituitaries injected over three days with 0·2 ml oil, 25 µg oestradiol benzoate (EB) or 3 mg tamoxifen (TX) and incubated for three hours with either medium alone, 10^{-8} M 17β-oestradiol (E_2) or 10^{-7} M TX in response to two consecutive 15-min GnRH challenges (10^{-8} M) at the beginning of the second and third hours of incubation. Values for LH secretion of hemipituitaries from oil- and from EB-injected OVX rats incubated with medium alone, E_2 or TX (24 hemipituitaries each), and those from TX-injected OVX rats incubated with medium alone or TX (16 hemipituitaries each) are represented together since no effect of the incubation conditions was found (see Table 1). Values for LH secretion from TX-injected OVX rats incubated with E_2 are the mean of 8 hemipituitaries. Values are means ± S.E.M. [a]Oestrogen environment increased basal and GnRH-stimulated LH secretion ($P<0·05$; ANOVA and Student-Newman-Keuls multiple range test).

Results

Experiment 1: E_2 inhibits TX-induced GnRH self-priming

The basal and GnRH-stimulated LH release increased in pituitaries from EB-treated, but not from TX-treated OVX rats (Fig. 1, Table 1). This increase was observed regardless of the incubation treatment applied. GnRH self-priming was observed both in EB- and TX-treated OVX rats (Figs 1 and 2). In EB-treated OVX rats, GnRH self-priming was observed in the three different incubation conditions (Fig. 2). In TX-treated OVX rats, however, GnRH self-priming was observed in pituitaries incubated with both medium alone and with TX but not in pituitaries incubated with E_2 (Fig. 2).

Experiment 2: the dose-dependent inhibitory effect of E_2 on TX-induced GnRH self-priming is steroid specific

TX-induced GnRH self-priming was abolished in a concentration-dependent manner by E_2. Thus, while 10^{-6}, 10^{-8} and 10^{-10} M E_2 inhibited GnRH self-priming, 10^{-12} M had no effect. Furthermore, addition of 10^{-8} M of the isomer oestradiol-17α to the medium did not influence TX-induced GnRH self-priming (Fig. 3). While coincubation with 10^{-7} M of the anti-oestrogen type II, ICI 182,780, reversed the inhibitory effect of E_2 on TX-induced GnRH self-priming, 10^{-7} M of the anti-oestrogen type I, TX, added to the incubation medium did not. Neither ICI 182,780 nor TX alone added to the medium had any effect (Fig. 4). The selective ERβ agonist, DPN, had no suppressive activity on TX-induced GnRH self-priming at any of the doses tested

Table 1 LH response (ng LH/ml) of incubated pituitary glands from two-week OVX rats injected (*in vivo* treatment) over three days with 0.2 ml oil, 25 µg estradiol benzoate (EB) or 3 mg tamoxifen (TX) and incubated (*in vitro* treatment) with medium alone, 10^{-8} M 17β estradiol (E_2) or 10^{-7} M TX, to two consecutive 15 min GnRH (10^{-8} M) pulses 1 h apart at the beginning of the second and third hours of incubation. Values are means ± S.E.M. of 8 hemipituitaries

		Basal LH	Peak LH response to 1st. GnRH pulse	Peak LH response to 2nd GnRH pulse
***In vivo* treatment**	***In vitro* treatment**			
Oil	Medium	28·6 ± 3·3	47·3 ± 3·5	50·7 ± 5·3
Oil	E_2	29·2 ± 2·2	44·5 ± 6·0	42·1 ± 6·2
Oil	TX	27·7 ± 2·0	35·8 ± 3·2	37·7 ± 6·2
EB	Medium	43·7 ± 3·4[a]	98·7 ± 8·1[a]	174·3 ± 18·8
EB	E_2	36·3 ± 2·9[a]	100·2 ± 10·7[a]	184·3 ± 19·2
EB	TX	39·1 ± 6·5[a]	94·6 ± 6·0[a]	168·3 ± 11·0
TX	Medium	33·9 ± 6·0	58·3 ± 10·1	109·5 ± 12·3
TX	E_2	23·2 ± 6·7	49·7 ± 6·8	48·7 ± 4·9
TX	TX	27·3 ± 5·6	52·6 ± 6·4	100·7 ± 10·8

[a]EB treatment increased both basal and GnRH-stimulated LH secretion ($P<0·05$) regardless of incubation conditions (ANOVA and Student–Newman–Keuls multiple range test).

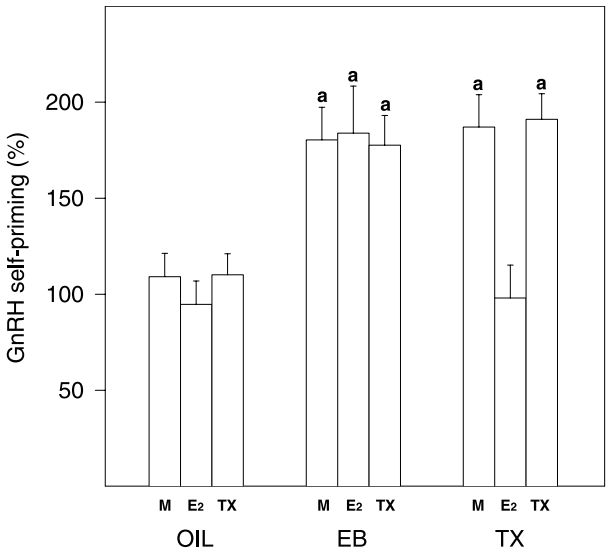

Figure 2 Mean ± S.E.M. values (8 hemipituitaries/group) for GnRH self-priming in hemipituitaries from OVX rats injected over three days with 0·2 ml oil, 25 µg oestradiol benzoate (EB) or 3 mg tamoxifen (TX) and incubated for three hours (1 h of preincubation + 2h of GnRH tests) with either medium alone (M), 10^{-8} M 17β-oestradiol (E_2) or 10^{-7} M TX. LHRH (10^{-8} M) was added to the incubation medium for 15 min at the beginning of the first (priming) and second (primed) hour of incubation. GnRH self-priming = peak response to the second LHRH pulse × 100/peak response to the first LHRH pulse. A value of 100% or less indicates absence of GnRH self-priming. $^aP<0{\cdot}05$ vs oil controls (ANOVA and Student-Newman-Keuls multiple range test).

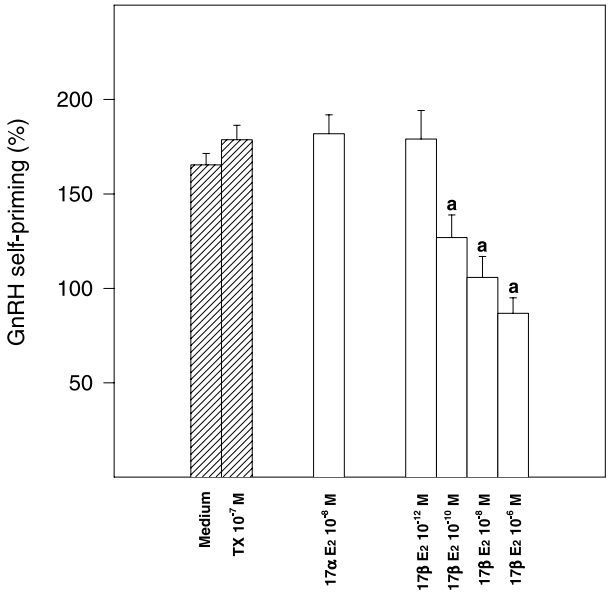

Figure 3 Effect of 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} M E_2 and 10^{-8} M 17α-oestradiol (17α E_2) on TX-induced GnRH self-priming. See legends of Fig. 1 for details of TX treatment and Fig. 2 for details of GnRH self-priming test. $^aP<0{\cdot}05$ vs controls (ANOVA and Student-Newman-Keuls multiple range test). Controls (TX-injected OVX rats incubated with or without 10^{-7} M TX; shaded bars) are the means of 32 hemipituitaries each of GnRH self-priming from experiments 2 and 3.

(Fig. 5). In contrast, 10^{-6} M, but not 10^{-8} or 10^{-10} M, of the selective ERα agonist, PPT, inhibited the GnRH self-priming induced by TX treatment (Fig. 5).

Experiment 3: E_2 appears to inhibit TX-induced GnRH self-priming by acting at the membrane surface

The 17β-oestradiol-BSA conjugate at a concentration of 10^{-6} M inhibited TX-induced GnRH self-priming, while 10^{-8} and 10^{-10} M did not (Fig. 6). Addition of 10^{-8} M E_2 to the medium over 15, 30, 45 or 60 min immediately before the first LHRH challenge inhibited TX-induced GnRH self-priming (Fig. 6).

Discussion

Treatment of OVX rats with TX induced GnRH self-priming without affecting the direct releasing action of GnRH (Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003). TX-induced GnRH self-priming is exerted through its high affinity and specificity binding to intracellular ER (McDonnell *et al.* 2002) by modulating the transcription of genes inducing progesterone receptor (PR) expression (Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003). This is because the agonistic action of TX is blocked by: (i) the simultaneous *in vivo* administration of RU58668 (Bellido *et al.* 2003), a pure anti-oestrogen (Vagell & McGinnis 1997); (ii) the anti-progesterone type II, RU486, both *in vivo* and *in vitro* (Bellido *et al.* 2003); and (iii) the anti-progesterone type I, ZK299, *in vitro* (Sánchez-Criado *et al.* 2005). The present incubation experiments showed, first, that none of the *in vitro* test substances significantly altered the first LH peak (data not shown), and secondly, that *in vivo* treatments fully determined the *in vitro* secretory response of pituitaries with one exception. Thus, the addition of E_2 or TX to the medium affected neither LH release nor GnRH self-priming in pituitaries from OVX rats injected with oil or EB. However, when E_2 was added to the medium of pituitaries collected from TX-treated OVX rats, the magnitude of the first LH peak was not changed but the height of the second LH peak was reduced, resulting in suppression of TX-induced GnRH self-priming. The finding that the agonist action (GnRH self-priming) of the antagonist TX was antagonized by the cognate agonist is intriguing. This hitherto-undescribed detrimental effect of E_2 on GnRH self-priming was steroid specific to the TX-treated OVX rats, since the concentration-dependent inhibitory effect of E_2 was not displayed by oestradiol-17α, and E_2 did not exhibit any inhibitory effect on LH secretion parameters in pituitaries

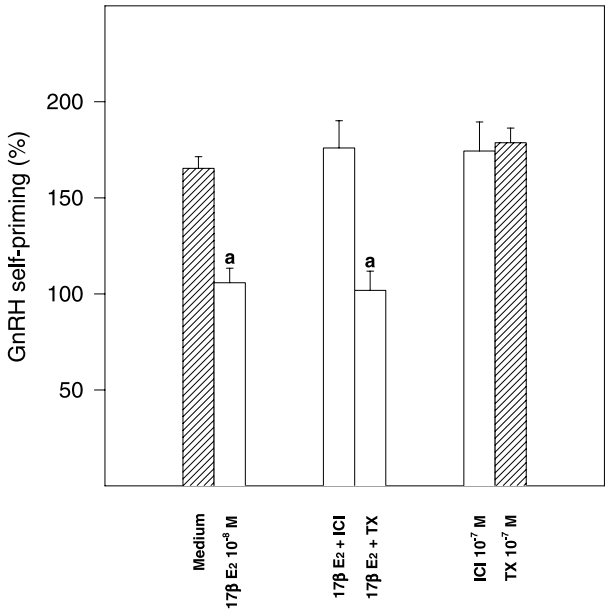

Figure 4 Effect of 10^{-7} M ICI 182,780 (ICI) or TX on E_2 inhibition of TX-induced GnRH self-priming. See legends of Fig. 1 for details of TX treatment, Fig. 2 for details of GnRH self-priming test, and Fig. 3 for details of control groups (shaded bars). There were eight hemipituitaries/group. $^aP<0{\cdot}05$ vs controls (ANOVA and Student-Newman-Keuls multiple range test).

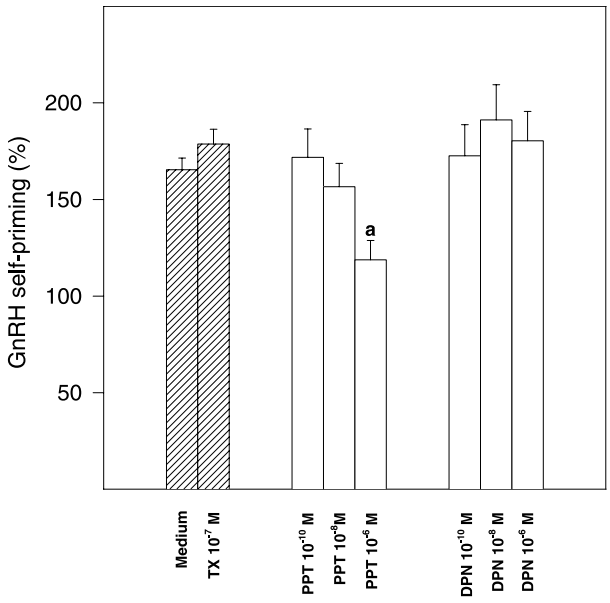

Figure 5 Effect of 10^{-10}, 10^{-8}, 10^{-6} M PPT or DPN on TX-induced GnRH self-priming. See legends of Fig. 1 for details of TX treatment, Fig. 2 for details of GnRH self-priming test, and Fig. 3 for details of control groups (shaded bars). There were eight hemipituitaries/group. $^aP<0{\cdot}05$ vs controls (ANOVA and Student-Newman-Keuls multiple range test).

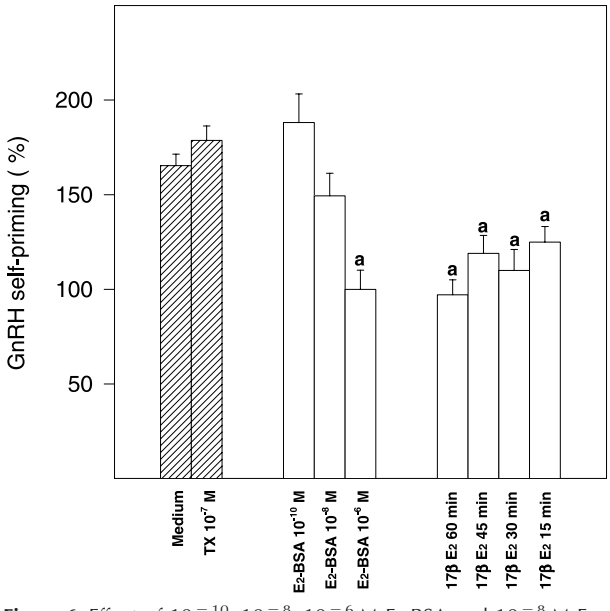

Figure 6 Effect of 10^{-10}, 10^{-8}, 10^{-6} M E_2-BSA and 10^{-8} M E_2 (17β E_2) during the last 15, 30, 45 and 60 min of the preincubation period on TX-induced GnRH self-priming. See legends of Fig. 1 for details of TX treatment, Fig. 2 for details of GnRH self-priming test, and Fig. 3 for details of control groups (shaded bars). There were eight hemipituitaries/group. $^aP<0{\cdot}05$ vs controls (ANOVA and Student-Newman-Keuls multiple range test).

collected from oil- or EB-treated OVX rats. It is worth highlighting the fact that the inhibitory effect of E_2 on TX-induced GnRH self-priming was reversed by coincubation with ICI 182,780 but not with TX. ICI 182,780 is a pure anti-oestrogen (type II) (Sun *et al.* 1999, Smith & O'Malley 2004) that blocks oestrogen binding to all known ERs (Leondires *et al.* 1999, McEwen & Alves 1999). Thus, E_2 appeared to inhibit the effect of TX on gonadotrophs acting on an ER with both high affinity for the anti-oestrogen type II ICI 182,780 and very low affinity for the anti-oestrogen type I TX. For all these reasons, it seems that the inhibitory action of E_2 was exerted at an ER different from the classical ER.

Although the isoform oestrogen receptor α predominates (Scully *et al.* 1997, Torand-Allerand 2004), the gonadotroph expresses both ERα and ERβ (Mitchner *et al.* 1998, Vaillant *et al.* 2002, Sánchez-Criado *et al.* 2005). In the present experiments, activation of ERβ with the specific agonist DPN (Meyers *et al.* 2001) had no inhibitory action on TX-induced GnRH self-priming, while activation of ERα with increasing concentrations of the ERα potency-selective agonist PPT (Sun *et al.* 1999, Stauffer *et al.* 2000) reduced it. These findings indicate that the inhibitory action of E_2 on GnRH self-priming could be exerted through an ERα-like isoform. In the rat, selective activation of each ER isoform with novel non-steroidal selective ligands for ERs indicates that, whereas PPT mimics all effects of oestrogen on gonadotroph

function, including PR expression and GnRH self-priming, DPN induces PR expression not followed by GnRH self-priming (Sánchez-Criado *et al.* 2004). Since TX, in the absence of the cognate ligand, induces PR expression and GnRH self-priming (Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003), it may be assumed (Sánchez-Criado *et al.* 2004, 2005) that TX agonist activity is exerted through intracellular ERα.

Sources of evidence for the classification of an effect as a non-genomic event are the rapid (seconds to minutes) time course (Bression *et al.* 1986, Morley *et al.* 1992), the insensitivity of the effect to inhibitors of transcription and protein synthesis (Pickering & Fink 1976) and the use of steroids coupled to macromolecules which prevent the steroid from entering the cell (Schmidt *et al.* 2000). In the present study, incubation of pituitaries from TX-treated OVX rats with increasing concentrations of the cell impermeant E_2-BSA significantly decreased GnRH self-priming in a dose-dependent manner. It is to be noted that in the present experiments all incubations were carried out in DMEM containing 0·1% BSA – a fact that ruled out potential BSA effects on LH pituitary response. In addition, incubation with E_2 for only 15 min, the shortest period possible in the present experimental design, had the same inhibitory effects as a two-hour incubation. Thus, although not decisive, these results are suggestive of a non-genomic event involved in the inhibitory effect of E_2 on the GnRH self-priming observed in pituitaries harvested from TX-treated OVX rats.

The inhibitory effect of physiological concentrations of *in vitro* E_2 on the agonist action of pharmacological doses of *in vivo* TX could be exerted through an isoform α-like ER exhibiting extremely low affinity for TX and located, presumably, in the plasma membrane of the gonadotroph. In physiological conditions, this inhibitory action of E_2 may have been masked by the simultaneous activation of the complete orchestra of ERs by the cognate ligand (McDonnell 2003). The physiological relevance of this hypothesis is not yet understood, but it would imply the existence of cross-talk between membrane and nuclear ERs in the gonadotroph to modulate E_2 action on GnRH self-priming and hence on the LH surge.

The activation of TX-induced PR in a ligand-independent manner (Blaustein 2004) is fundamental for GnRH self-priming to occur, as TX-induced GnRH self-priming can be suppressed by the progesterone antagonists RU486 (Bellido *et al.* 2003) or ZK299 (Sánchez-Criado *et al.* 2005) in the absence of progesterone. Accordingly, if the interpretation of the present results were correct, it would be tempting to speculate that the inhibitory action of E_2 on TX-induced GnRH self-priming affects the interaction between GnRH intracellular signals, protein kinase (PK) A (Waring & Turgeon 1992, Turgeon & Waring 1994) or PKC (Fink 1995, Aguilar *et al.* 2003), and the TX-dependent PR (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2005).

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