Oestradiol-17β inhibits tamoxifen-induced LHRH self-priming blocking hormone-dependent and ligand-independent activation of the gonadotrope progesterone receptor in the rat

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Abstract

In the rat, administration of tamoxifen (TX) in the absence of oestrogen (E) induces LHRH self-priming, the progesterone receptor (PR)-dependent property of LHRH that increases gonadotrope responsiveness to itself. The oestrogen-dependent PR can be phosphorylated/activated by progesterone (P_4) and, in the absence of the cognate ligand, by intracellular LHRH signals, particularly cAMP/protein kinase A. We have recently found that oestradiol-17β (E_2), acting on a putative membrane estrogen receptor-α in the gonadotrope, inhibits this agonist action of TX. This study investigated the mechanism by which E_2 inhibits TX-elicited LHRH self-priming using both incubated pituitaries from TX-treated ovariectomized (OVX) rats and anterior pituitary cells from OVX rats cultured with TX. It was found that (1) in addition to the inhibitory effect on TX-elicited LHRH self-priming, E_2 blocked P_4 and adenylyl cyclase activator forskolin augmentation of LHRH-stimulated LH secretion, and (2) E_2 did not affect the increasing action of TX on gonadotrope PR expression or pituitary cAMP content. Furthermore, inhibition of protein phosphatases with okadaic acid suppressed E_2 inhibition of TX-elicited LHRH-induced LH secretion, while stimulation of protein phosphatases with ceramide blocked TX-induced LHRH self-priming. Together, these results indicated that membrane ER-mediated E_2 inhibition of the TX-stimulated LHRH self-priming pathway involves a blockade of gonadotrope PR phosphorylation/activation, but not a deficient response of PR to phosphorylases. Results also suggested that the inhibitory effect of E_2 on TX-induced LHRH self-priming is exerted through modulation of cellular protein phosphatase activity in the gonadotrope.

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Introduction

In an oestrogen background, luteinizing hormone-releasing hormone (LHRH)-induced ovulatory luteinizing hormone (LH) release in the rat involves two pathways: (1) the releasing pathway, whereby LHRH causes directly the secretion of LH (Fink 1988) and (2) the self-priming pathway, the property of LHRH that increases responsiveness of the gonadotrope to itself (Pickering & Fink 1976, Waring & Turgeon 1983, Fink 2000, de Koning *et al.* 2001). The oestrogen-dependent LHRH self-priming pathway involves *de novo* synthesis of RNA and proteins (de Koning *et al.* 1976, Pickering & Fink 1976, Fink 1988, Turgeon & Waring 1991), the priming proteins (Fink 1995), and is, in turn, dependent on oestrogen-induced upregulation of progesterone receptor (PR) in gonadotropes (Turgeon & Waring 1992, Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003). Activation of the complete oestrogen receptor (ER) orchestra after 3-day administration of oestrogen in rats 2 weeks after ovariectomy (OVX) mimicked the endocrine events of pro-oestros through the augmentation of the LHRH-releasing pathway, progesterone (P_4) receptor (PR) expression, and PR-dependent LHRH self-priming (Bellido *et al.* 2003, Sánchez- Criado *et al.* 2004), culminating in an ovulatory LH surge (Legan & Tsai 2003).

In experiments to analyse the effects of selective ER modulators (SERMs) on gonadotropes using a similar OVX rat model, it was found that the triphenylethylene SERM tamoxifen (TX), but not the benzothiophene-derivative SERMs (Sánchez-Criado *et al.* 2002, 2005*a*), induced PR expression and LHRH self-priming without affecting the LHRH releasing pathway (Sánchez-Criado *et al.* 2002, 2004, Bellido *et al.* 2003). It has also been found that TX exerts these agonistic actions in the absence of oestrogen, most probably through binding nuclear ER-α (Sánchez-Criado *et al.* 2004, 2005*a*, 2006). Interestingly, the ER cognate agonist oestradiol-17β (E_2) added to the incubation medium

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of pituitaries, collected from TX-treated OVX rats, inhibits TX-induced LHRH self-priming in a dose-dependent manner (Bellido *et al.* 2005, Sánchez-Criado *et al.* 2005*b*). Further, relevant findings on this inhibitory effect of E_2 are: (a) the pure anti-oestrogen ICI182,780, but not the TX itself, reverses the inhibitory action of E_2; (b) the ER-α selective agonist propylpyrazole triol (Stauffer *et al.* 2000), but not the ER-β selective agonist diarylpropionitrile (Meyers *et al.* 2001), mimics the inhibitory effect of E_2, and (c) the analogue membrane-impermeable conjugated E_2-BSA inhibits TX-induced LHRH self-priming, whereas the isomer E_2-17α does not. For all these reasons, it has been suggested that the inhibitory action of E_2 on TX-elicited LHRH self-priming takes place via activation of membrane ER-α exhibiting extremely low affinity for TX (Sánchez-Criado *et al.* 2005*b*).

Much work has been done on the interaction between membrane and intracellular ERs (Pedram *et al.* 2002, Levin 2005). The proposed non-genomic inhibitory action of E_2 on the genomic effect of TX-eliciting LHRH self-priming is a good model to further explore this interaction at the gonadotrope level. Oestrogen-dependent LHRH self-priming is a consequence of PR phosphorylation/activation by P_4 (Turgeon & Waring 1990, Levine 1997) or by intracellular LHRH signals, particularly cAMP/protein kinase A (PKA) (Turgeon & Waring 1992, 1994, 2001) in a ligand-independent manner (Weigel & Zhang 1994, Cenni & Picard 1999, Blaustein 2004). The dynamic balance between phosphorylation and dephosphorylation rates determines the activation of the PR (Zhang *et al.* 1994). Using anterior pituitaries from TX-treated OVX rats as well as TX-cultured anterior pituitary cells from OVX rats, this study investigated the action of E_2 on: (1) LHRH self-priming; (2) the potentiating effect of P_4 and adenylyl cyclase activator forskolin (FSK) on LHRH-induced LH secretion; (3) gonadotrope PR expression and pituitary cAMP content and (4) intracellular protein phosphatase activity.

Materials and Methods

Animals, treatments and experimental groups

Adult female Wistar rats weighing 190–210 g were used. Rats were housed under a 14 h light:10 h darkness cycle (light on at 0500 h) and 22±2 °C room temperature, with access to rat chow and tap water *ad libitum*. All rats were ovariectomized (OVX) under light ether anaesthesia at random stages of the oestrous cycle and were used 2 weeks later. For incubation studies of anterior hemipituitaries, rats were injected daily at 0900 h over 3 days with 3 mg TX (Sigma). The day after treatment, rats were decapitated and anterior pituitary glands dissected out and divided into halves. One half was incubated for 3 h with 10^{-7} M TX (TX+TX) and the other half with 10^{-8} M E_2 (TX+E_2) (Sigma). Control groups consisted of (i) hemipituitaries from OVX rats injected over 3 days with 0.2 ml oil and incubated with medium alone (oil+Dulbecco's modified Eagle's medium (DMEM); Sigma) and (ii) hemipituitaries from OVX rats injected daily over 3 days with 25 μg oestradiol benzoate (EB) (Sigma) and incubated with 10^{-8} M E_2 (EB+E_2). For cultured pituitary cell studies, pituitary cells from OVX rats were cultured for 72 h with 10^{-7} M TX, then for 3 h either with 10^{-7} M TX (TX+TX) or 10^{-8} M E_2 (TX+E_2). As in incubation studies, control pituitary cells were incubated for 75 h with medium alone (DMEM+DMEM) or 10^{-8} M E_2 (E_2+E_2) (Fig. 1).

All pituitary incubation and cell culture experiments lasted for 3 h. During the first hour, no secretagogues were added, this being considered a period of basal LH secretion. During the second hour, priming agents were added; this was considered the phase during which 'priming proteins' (Fink 1995) are expressed (priming hour). Finally, during the third hour, LHRH (Peninsula Laboratories, Inc., St Helens, Merseyside, UK) was added and the pituitary responsiveness measured (primed hour) (Fig. 1). Doses of hormones and drugs used and timing of treatments come from previous papers (Denner *et al.* 1990, Dobrowsky & Hannun 1992, Turgeon & Waring 1991, 1992, Waring & Turgeon 1992, Sánchez-Criado *et al.* 2002, 2004, 2006).

Incubation procedure for hemipituitaries

Incubation of hemipituitaries was carried out as previously described (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). Briefly, hemipituitaries were incubated after 1 h of pre-incubation 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 DMEM, without L-glutamine and phenol red, containing glucose (4·5 g/l) and BSA 0.1%, w/v, pH 7·4.

Pituitary cell dispersion and primary culture protocol

Anterior pituitary glands were immersed in DMEM without phenol red (Sigma), containing 0·3% BSA, 25 mM Hepes and buffered with 20 mM $NaHCO_3$ to pH 7·4. Dispersion was carried out as previously described (Dobado-Berrios *et al.* 1996). Briefly, pituitaries were minced and sequentially incubated in 0·2% trypsin (type I), and Ca^{2+}/Mg^{2+}-free Hanks' balanced solution containing 2 and 1 mM EDTA, followed by a short mechanical dissociation. The cell suspension was then centrifuged and re-suspended in DMEM. Cell yield and viability were estimated by the Trypan Blue exclusion test.

Dispersed pituitary cells were cultured (37 °C, 95% air, 5% CO_2) in 24-well tissue culture plate at a density of 3×10^5 cells/well in 0·5 ml DMEM alone or with test substances, and supplemented with 10% charcoal-stripped foetal bovine serum (FBS) and gentamycin sulphate (50 μg/ml). After 48 h of culture, media was replaced with fresh media. Experiments were carried out after 72-h culturing. A 1-h preincubation in fresh DMEM without FBS was used to stabilize basal LH secretion before adding test substances.

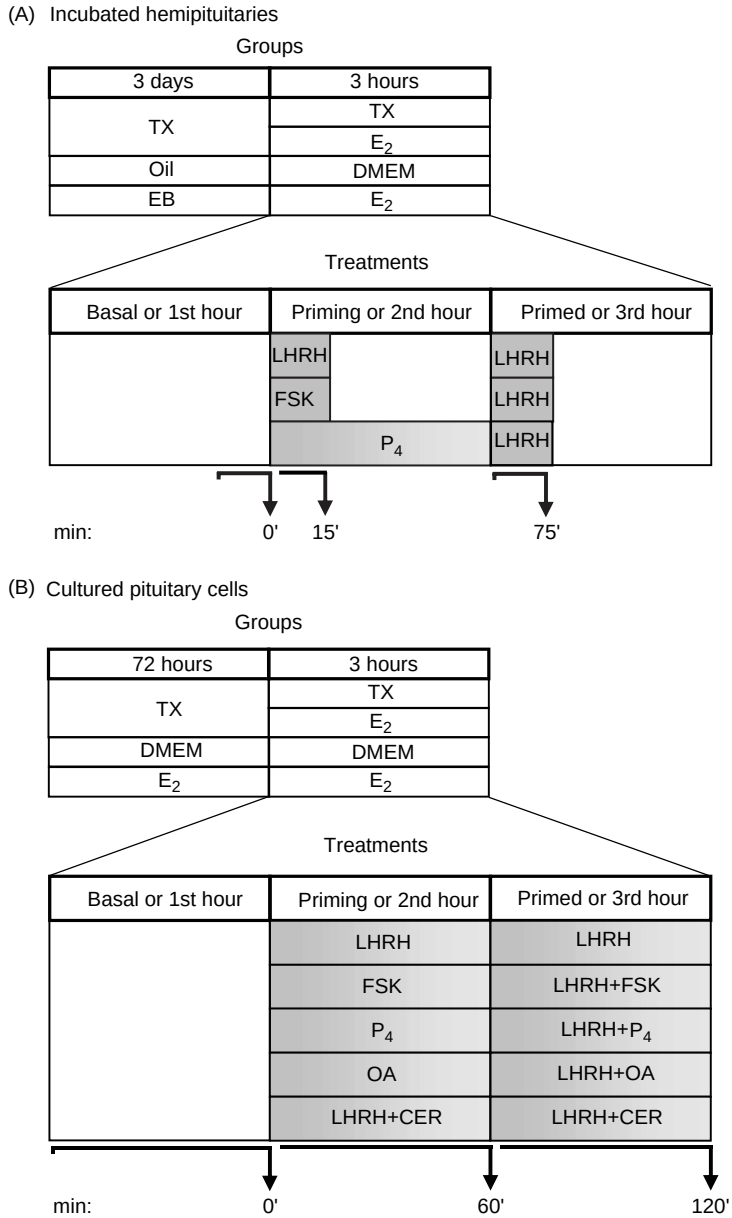

Figure 1 Diagram showing the experimental groups, type of treatment and its duration (shaded areas), and the time of collection of media for determination of LH concentration (arrows) during the 3 h: basal (first hour), priming (second hour) and primed (third hour) of the experiments both in (A) incubated hemipituitaries and (B) cultured pituitary cells. All treatments were applied to experimental and control groups either in incubated hemipituitaries (TX + TX, TX + E_2, oil + DMEM and EB + E_2) or cultured pituitary cells (TX + TX, TX + E_2, DMEM + DMEM and E_2 + E_2). See Materials and Methods for details of treatments. LHRH = 10^{-8} M luteinizing hormone-releasing hormone, FSK = 5×10^{-5} M forskolin, P_4 = 10^{-7} M progesterone, OA = 5×10^{-8} M okadaic acid, CER = 10^{-7} M ceramide. In experiments with incubated hemipituitaries (A), LH accumulation was determined during the first 15 min of the priming and primed hours. The last 15 min collection during the first hour was used to determine basal LH secretion. In experiments with cultured pituitary cells (B), LH accumulation was determined in the whole medium at the end of each of the 3 h.

At least three separate independent experiments were performed and a minimum of four replicate wells per treatment were tested in each experiment.

Experimental treatment of incubated hemipituitaries

Two different sets of experiments were performed, the first to evaluate LHRH self-priming by LH measurement using RIA, and the second to determine PR expression by immunohistochemistry and cAMP content by immunoassay in the pituitary.

Hemipituitaries from control (oil+DMEM and EB+E_2) and experimental (TX+TX and TX+E_2) groups were primed (second hour) with LHRH (Peninsula Laboratories, Inc., St Helens, Merseyside, UK), the adenylyl cyclase activator FSK (Sigma) or P_4 (Sigma). Priming treatments consisted of exposure of the hemipituitaries to 10^{-8} M LHRH or 5×10^{-5} M FSK for the first 15 min of the priming hour or to 10^{-7} M P_4 for the entire hour of priming. All groups were challenged with a second 15-min pulse of 10^{-8} M LHRH at the beginning of the primed hour (third hour). All medium was aspirated at the end of the first 15 min of both the priming and the primed hours for quantification of LH concentration and replaced with fresh medium. The last 15-min collection during the basal hour (first hour) was used to estimate basal LH secretion (Fig. 1A).

To determine PR expression and cAMP content in pituitaries during the priming hour, hemipituitaries from 22 rats/group (oil+DMEM, EB+E_2, TX+TX and TX+E_2) were incubated during the first hour as described. Then they were either primed or not with 10^{-8} M LHRH during the first 15 min of the priming hour (second hour). Finally, hemipituitaries were removed and processed for PR localization or cAMP content at 15 and 60 min of the priming hour.

Experimental treatment of cultured pituitary cells

Two different experiments were performed, the first to evaluate LHRH self-priming by LH measurement using RIA, and the second to determine the effect of activation and inhibition of intracellular phosphatases on LHRH-stimulated LH secretion.

After 72-h culture with 10^{-7} M TX, these treated pituitary cells were cultured for a further 3-h period throughout the basal, priming and primed hours with either TX (TX+TX) or 10^{-8} M E_2 (TX+E_2). Control pituitary cells were incubated for 75 h in either medium alone (DMEM+DMEM) or 10^{-8} M E_2 (E_2+E_2). Priming treatments in the two control and experimental groups included 10^{-8} M LHRH over the whole priming hour and 5×10^{-5} M FSK or 10^{-7} M P_4 during the priming and primed hours. Finally, 10^{-8} M LHRH was added during the primed hour to evaluate pituitary responsiveness. The whole medium was removed every hour, replaced with fresh medium and assayed for LH concentration (Fig. 1B).

Using the pituitary cell culture system described earlier and the same experimental (TX+TX, TX+E_2) and control (DMEM+DMEM, E_2+E_2) groups, the effect of inhibiting with okadaic acid (OA) or activating with ceramide (CER) intracellular phosphatases on E_2-induced inhibition of TX-induced LHRH self-priming was tested. On the first study 5×10^{-8} M OA (Sigma), a potent inhibitor of protein phosphatases 1 and 2 (Denner *et al.* 1990), was added to the medium during both the priming and primed hours, and 10^{-8} M LHRH was added during the primed hour to evaluate the LH response. In the second study, anterior pituitary cells were co-incubated with LHRH and 10^{-7} M D-erythro-sphingosine, *N*-acetyl-C_2 CER (Sigma), an activator of protein phosphatases 2A (Dobrowsky & Hannun 1992) during the priming and primed hours (Fig. 1B).

LHRH self-priming evaluation

LHRH self-priming was considered to be present when the magnitude of the LH response to the second of two exposures to LHRH (primed hour) was significantly greater than the unprimed response (priming hour). In incubation studies, the peak pituitary LH response to a 15-min LHRH challenge occurs after 15 min exposure to the decapeptide (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). In cell culture studies, LHRH self-priming or potentiation of LHRH secretory action elicited by priming substances (FSK, P_4) were evaluated by statistical comparison between LH accumulation in the medium during the 1-h primed hour and the 1-h priming hour.

RIA of LH

LH concentrations 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 (Sánchez-Criado *et al.* 2004). Rat LH-I-10 was labelled with ^{125}I by the chloramine T method (Greenwood *et al.* 1963). Intra- and interassay coefficients of variation were 8 and 9% respectively. Assay sensitivity was 3.75 pg/tube. LH concentration was expressed as nanograms per hemipituitary or nanograms per 3×10^{-5} cells of the reference preparation LH-rat-RP-3.

Immunohistochemistry of pituitary PR

The immunohistochemical study was performed on dewaxed and rehydrated 3 μm thick sections of formalin-fixed, paraffin-embedded tissue samples. The commercial mouse monoclonal anti-human PR antibody clone PR10A9, raised against the recombinant hormone-binding domain of human PR located on the C-terminal domain of PR (Immunotech, Marseille, France), was diluted in the ratio of 1:15 000 and used in the avidin biotin peroxidase complex technique (Vector, Burlingame, CA, USA) as previously described (Sánchez-Criado *et al.* 2004, 2006). Tissue sections from similarly processed samples of rat

uterus and human breast carcinoma were used as positive controls. The specificity of the PR antibody was shown by the absence of staining after preincubation of tissue sections of rat uterus and pituitaries from OVX rats treated with EB with 10^{-9}, 10^{-7} and 10^{-5} M of P_4 for 1 h at 37 °C. Substitution of the specific primary antibody by mouse ascitic fluid at the same dilution as the specific primary antibody in tissue sections of the cases under study was used as negative control. Several dilutions of the PR10A9 monoclonal antibody were tested; the optimal dilution was established at 1:15 000, which provided the highest intensity of nuclear staining with the lowest background staining of pituitary and uterus (Sánchez-Criado *et al.* 2004). Nuclear counterstaining was performed with Mayer's haematoxylin in all cases. The number of cells immunoreactive to PR antibody was counted in 15 high-power fields (×40) per pituitary tissue section (five sections from each of the eight rats per group) and is expressed as number of positive nuclei/200 μm^2. Labelling intensity was evaluated on a 2-point scale as deep brown and medium brown.

cAMP determination

All pituitaries from the two experimental (TX + TX, TX + E_2) and control (oil + DMEM, EB + E_2) groups were challenged for 15 min during the priming hour with LHRH. At 15 and 60 min of the priming hour, hemipituitaries were quick frozen at −80 °C until assayed for cAMP determination. cAMP content was determined as follows: hemipituitaries were homogenized by sonication (100 W, 5 s) in 200 µl of 0·05 M sodium acetate buffer pH 5·8 containing 0·02% BSA and boiled at 90 °C for 15 min. After centrifuging for 5 min at 2000 ***g***, supernatant was recovered for cAMP determination. cAMP measurements were made with the cAMP enzyme immunoassay kit (RP225) (Amersham). The detection limit was 12·5 fmol, and the intraassay variation coefficient was less than 7%. Data were expressed as femtomoles per microgram of total protein measured by the bicinchoninic acid method (Smith *et al.* 1985). Values were represented as percentage variation with respect to controls (rats injected with oil and incubated with DMEM alone). In these experiments, 1 µM dopamine was present during the incubation period in order to minimize the contribution of lactotrope cAMP (Martinez de la Escalera & Weiner 1988, Waring & Turgeon 1992).

Statistical analysis

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

Results

Effect of E_2 on the potentiating effect of LHRH, P_4 and FSK on LHRH-stimulated LH secretion

Secretion of LH from incubated hemipituitaries (Table 1) and cultured pituitary cells (Table 2) was similar, though minor

Table 1 LH response (ng/hemipituitary) of incubated pituitaries from 2-week OVX rats injected (*in vivo* treatment) with 3 mg tamoxifen (TX) daily for 3 days and, after 1-h pre-incubation, incubated for 3 h (*in vitro* treatment) either with 10^{-7} M TX or 10^{-8} M oestradiol-17β (E_2) to a 15-min LHRH (10^{-8} M) pulse during the primed hour after 1 h of priming. Control groups consisted of pituitaries from 0·2 ml oil-injected OVX rats incubated with medium alone and pituitaries from 25 µg oestradiol benzoate (EB)-injected OVX rats incubated with 10^{-8} M E_2. Hemipituitaries were primed with either 10^{-8} M LHRH or 5×10^{-5} M forskolin (FSK) during the first 15 min of priming hour, or 10^{-7} M progesterone (P4) during the 60 min of the priming hour. Values are means ± S.E.M. of eight hemipituitaries

	LH (ng)/hemipituitary			
	In vitro (3 h)	Basal hour (last 15 min)	Priming hour (first 15 min)	Primed hour (first 15 min)
Treatment				
***In vivo* (72 h)**				
Oil	DMEM + LHRH	38·2 ± 3·4	50·2 ± 5·0	59·4 ± 3·0
EB	E_2 + LHRH	76·8 ± 4·2[a]	159·4 ± 23·3[a]	279·7 ± 35·2[b]
TX	TX + LHRH	43·7 ± 8·4	71·4 ± 22·4	147·8 ± 12·8[b]
TX	E_2 + LHRH	56·9 ± 8·2	57·7 ± 8·2	60·8 ± 12·8[d]
Oil	DMEM + FSK	44·1 ± 4·9	38·3 ± 3·2	58·6 ± 4·1
EB	E_2 + FSK	73·3 ± 9·4[a]	82·8 ± 8·1	265·5 ± 20·0[c]
TX	TX + FSK	44·9 ± 9·2	55·2 ± 6·3	165·5 ± 16·1[c]
TX	E_2 + FSK	43·6 ± 5·2	50·4 ± 10·9	65·1 ± 10·9[d]
Oil	DMEM + P4	37·9 ± 3·7	42·2 ± 5·8	59·4 ± 5·7
EB	E_2 + P4	82·6 ± 8·2[a]	79·8 ± 10·0	285·1 ± 29·1[c]
TX	TX + P4	55·0 ± 10·8	51·2 ± 13·0	150·8 ± 13·3[c]
TX	E_2 + P4	59·9 ± 6·7	47·9 ± 2·8	48·0 ± 4·3[d]

[a]Basal and LHRH-stimulated LH secretion were increased ($P<0·05$) in an oestrogen environment (EB + E_2).
[b]LHRH self-priming was noted ($P<0·05$) in oestrogen and TX environments.
[c]P_4 and FSK priming induced potentiation of LHRH-stimulated LH secretion ($P<0·05$) in oestrogen and TX environments.
[d]Pituitaries from TX-treated rats incubated with E_2 did not exhibit LHRH self-priming nor P_4- or FSK-induced potentiation of LHRH-stimulated LH secretion. ANOVA and Student–Newman–Keuls multiple range test.

Table 2 LH response (ng/3×10^5 cells) of pituitary cells from 2-week OVX rats to 1-h 10^{-8} M LHRH during the primed hour after 1 h of priming. Cells were incubated over 72 h with 10^{-10} M TX and, after 1-h pre-incubation in fresh medium, treated either with TX or 10^{-8} M oestradiol-17β (E_2). Control groups consisted of pituitary cells from 2-week OVX rats incubated over 75 h with medium alone or 10^{-8} M E_2. Pituitary cells were treated during the priming and primed hours with 10^{-8} M LHRH, 10^{-7} M progesterone (P_4) or 5×10^{-5} M FSK. At least four replicates per experiment were tested. Values are means±S.E.M. of three independent experiments

	LH (ng)/3×10^5 cells			
	Experiment (3 h)	Basal hour	Priming hour	Primed hour
Treatment				
Incubation (72 h)				
DMEM	DMEM+LHRH	5·9±2·4	10·2±3·9	11·2±3·0
E_2	E_2+LHRH	7·7±1·2	15·3±2·2[a]	40·2±7·4[b]
TX	TX+LHRH	6·4±0·7	10·0±2·6	20·2±3·9[b]
TX	E_2+LHRH	8·0±2·3	8·3±1.6	10·5±1·1[d]
DMEM	DMEM+FSK	6·6±1·5	3·9±0.8	14·7±3·1
E_2	E_2+FSK	6·5±1·0	4·5±1.0	37·4±7·3[c]
TX	TX+FSK	4·7±0·9	3·6±0.8	18·2±4·5[c]
TX	E_2+FSK	7·2±2·3	3·6±1.0	3·6±0.5[d]
DMEM	DMEM+P4	7·0±1·3	5·0±0.8	14·2±3·2
E_2	E_2+P4	6·6±8·2	4·8±1.0	38·7±4·4[c]
TX	TX+P4	4·2±0·8	3·7±1.3	20·1±2·3[c]
TX	E_2+P4	4·8±0·9	2·7±1.0	7·7±1.3[d]

[a]LHRH-stimulated LH secretion was increased ($P<0·05$) in E_2-cultured pituitary cells.
[b]LHRH self-priming was noted ($P<0·05$) in E_2- and TX-treated pituitary cells.
[c]P_4 and FSK priming induced potentiation of LHRH-stimulated LH secretion ($P<0·05$) in E_2- and TX-treated pituitary cells.
[d]Addition of E_2 to the culture medium of TX-treated pituitary cells blocked the priming effect of LHRH, P_4 and FSK. ANOVA and Student–Newman–Keuls multiple range test.

differences in LH concentration were noticed during the basal hour. In an oestrogen background (EB+E_2 and E_2+E_2 groups), hemipituitaries and anterior pituitary cells displayed LHRH-stimulated LH release during the priming hour and LHRH self-priming in the primed hour. In a TX background (TX+TX groups), hemipituitaries and anterior pituitary cells showed only LHRH self-priming and this effect of TX was annulled by addition of E_2 to the medium (Tables 1 and 2). Exposure of control (EB+E_2 and E_2+E_2) and experimental (TX+TX) groups to P_4 or FSK during the priming hour did not elicit LH release during the priming hour, but potentiated LHRH-induced secretion of LH during the primed hour. Addition of E_2 to the medium of hemipituitaries or pituitary cells annulled this effect in the TX+TX groups, but not in the oestrogen-treated groups (EB+E_2 and E_2+E_2) (Tables 1 and 2).

Effect of E_2 on PR expression and cAMP content in hemipituitaries from TX-treated OVX rats

The number of pituitary cells immunoreactive to PR, and the intensity of PR staining, were similar in pituitaries incubated for 15 or 60 min during the priming hour and LHRH did not affect either the extent or the intensity of PR staining. In view of these findings, PR expression values are shown together in Fig. 2 (12 hemipituitaries/group). Panel (A) shows representative examples of the effects of ER ligands on pituitary cell morphology and PR expression. Treatment with ER ligands appeared to induce shrinkage of gonadotropes and upregulated PR expression in OVX rats (Fig. 2A). The lower panel shows that both the number of pituitary cells immunoreactive to PR and the intensity of PR staining were significantly higher in oestrogen-treated pituitaries than in TX-treated pituitaries. Finally, incubation of pituitaries from TX-treated rats with E_2 had no effect on the extent or intensity of PR expression (Fig. 2B).

In EB-treated rats incubated with E_2, LHRH increased the pituitary cAMP content at the end of the 15-min challenge in the priming period (Fig. 3A). However, this pituitary response to LHRH was ephemeral, since no stimulating effect of LHRH on cAMP pituitary content was noted at the end of the priming hour (Fig. 3B). In TX-treated rats, pituitary cAMP content was significantly increased over that of hemipituitaries from controls (oil+medium) during the entire priming period (15 and 60 min) regardless of the presence of E_2 in the medium (TX+TX vs TX+E_2 groups). Unexpectedly, however, no increase in cAMP content was observed in response to LHRH in the TX-treated rats (Fig. 3).

Effect of activation or inhibition of intracellular phosphatases on E_2-inhibited TX-induced LHRH self-priming

Stimulation of intracellular protein phosphatases with CER annulled LHRH-stimulated LH secretion in E_2-treated anterior pituitary cells, and reduced and blocked LHRH self-priming in E_2- and TX-treated pituitary cells respectively. By contrast, CER had no significant inhibitory effect on any

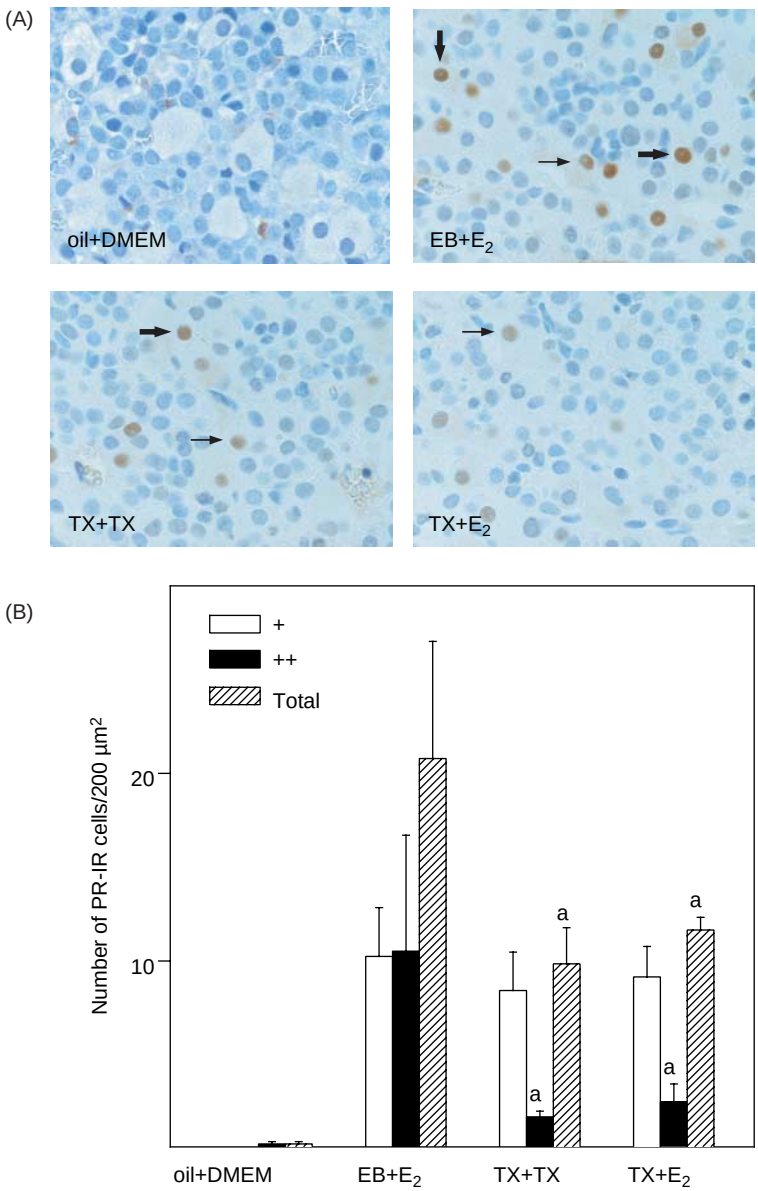

Figure 2 PR immunoreactivity at 15 and 60 min of the priming hour in hemipituitaries from OVX rats injected with 3 mg tamoxifen (TX) daily for 3 days and incubated with either 10^{-7} M TX (TX+TX; $n=6$) or 10^{-8} M oestradiol-17β (TX+E_2; $n=6$). Control groups consisted of hemipituitaries from OVX rats injected with 0.2 ml oil and incubated with medium alone (oil+DMEM; $n=6$) or with 25 µg oestradiol benzoate (EB) and incubated with E_2 (EB+E_2; $n=6$). Hemipituitaries in all groups were challenged ($n=3$) or not ($n=3$) with 10^{-8} M LHRH for the first 15-min of the priming hour. Since no differences were found in PR expression at 15 or 60 min incubation, and LHRH had no effect on PR immunoreactivity, values in the lower panel are means of 12 hemipituitaries/group. Several immunoreactive nuclei with deep brown ((A) thick arrows and (B) ++) and medium brown ((A) thin arrows and (B) +) staining intensities are shown. The upper images are representative examples of the control and experimental groups. ABC technique, nuclear counterstaining with Mayer's haematoxylin X40. a, $P<0{\cdot}05$ vs EB+E_2; ANOVA followed by the Student–Newman–Keuls multiple range test.

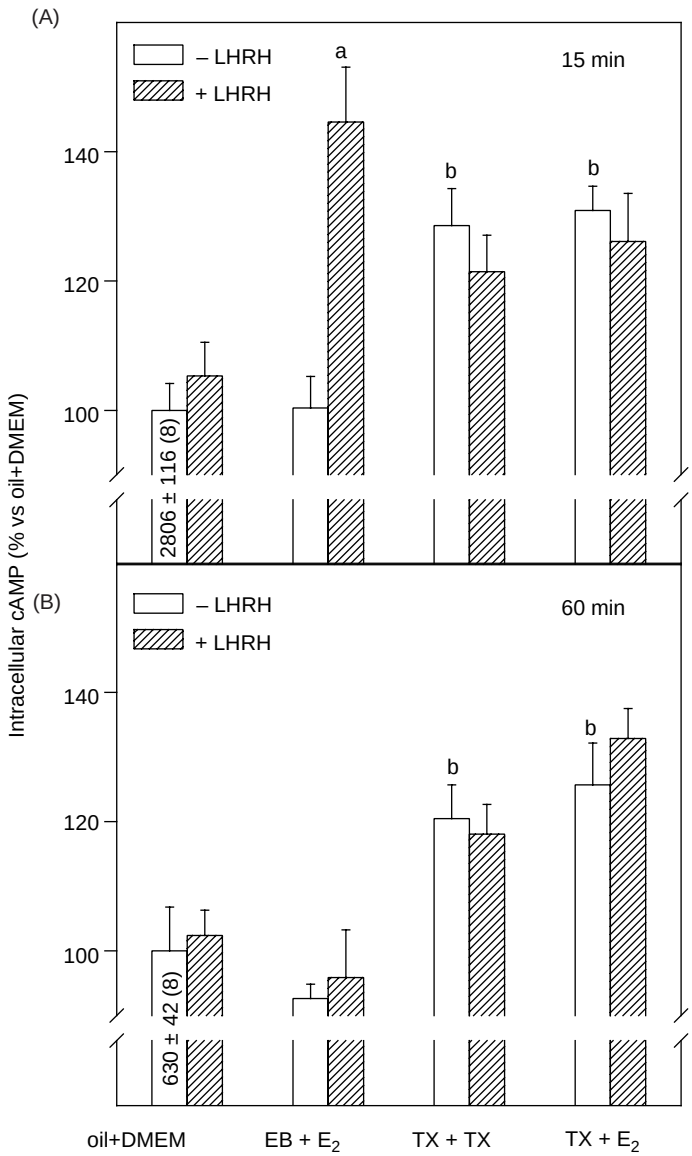

Figure 3 Intracellular cAMP content in hemipituitaries at (A) 15 and (B) 60 min of the priming hour in experimental (TX + TX and TX + E_2) and control (oil + DMEM and EB + E_2) groups. See Fig. 1 for details of treatments. Values are the mean of eight hemipituitaries and are expressed as percentage variation with respect to oil + DMEM group without LHRH. Values are denoted as mean ± S.E.M. (fmol/mg protein). All hemipituitaries were challenged (dashed bars) or not (open bars) with 10^{-8} M LHRH for the first 15 min of the priming hour. a, $P<0{\cdot}05$ vs non-stimulated hemipituitaries with LHRH. b, $P<0{\cdot}05$ vs oil + DMEM control group; ANOVA followed by the Student–Newman–Keuls multiple range test.

parameter of LH secretion in TX-treated pituitary cells exposed to E_2 (Fig. 4). On the other hand, inhibition of intracellular protein phosphatases with OA elicited LHRH-stimulated LH secretion in all groups of pituitary cells treated with ER ligands (Fig. 4). The response to LHRH in OA-treated cells was higher ($P<0{\cdot}05$) than that of pituitary cells to LHRH during the priming hour in E ($E_2 + E_2$) and TX (TX + TX) backgrounds.

Discussion

The present results show that the addition of E_2 to the incubation medium of TX-treated anterior pituitary cells blocks TX-induced LHRH self-priming, confirming previous observations using incubated pituitaries from TX-treated rats (Sánchez-Criado *et al.* 2002, 2005*b*, Bellido *et al.* 2003).

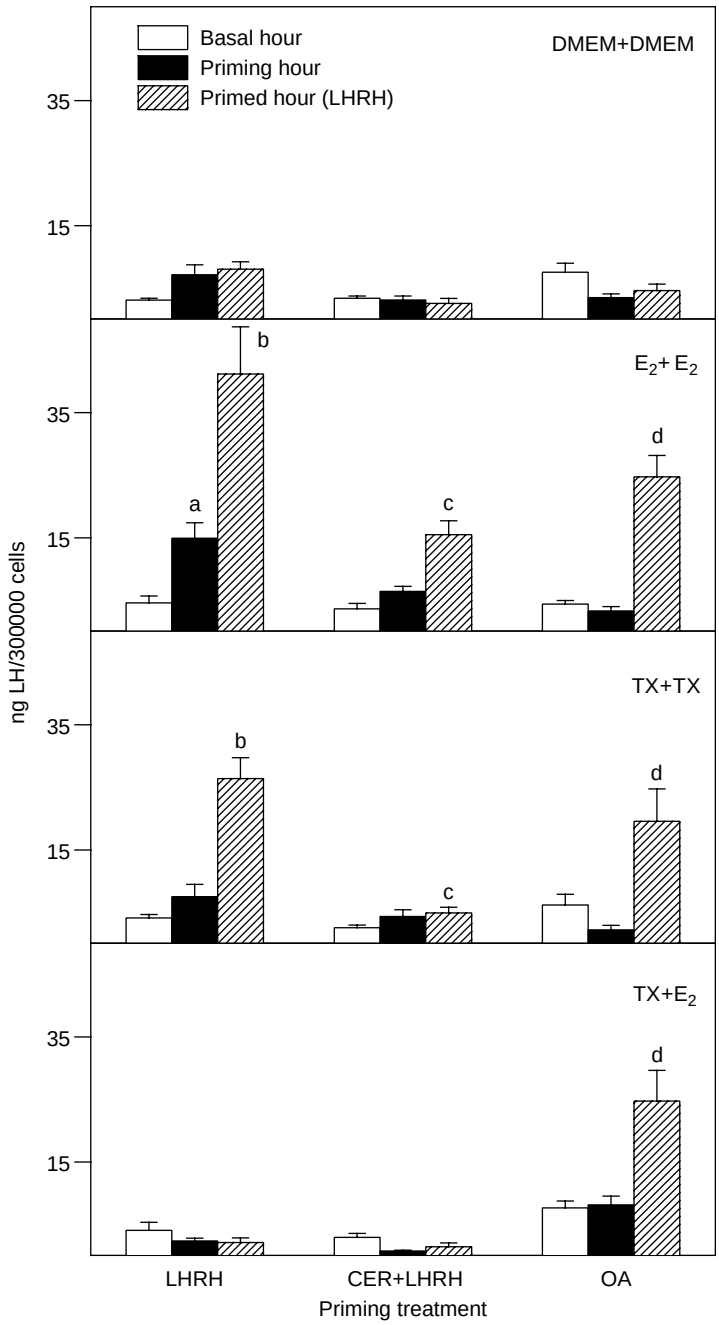

Figure 4 Effects of 10^{-7} M CER on LHRH self-priming elicited by 10^{-8} M LHRH, and 5×10^{-8} M OA on 10^{-8} M LHRH-induced LH secretion in pituitary cells culture. Cells were incubated over 72 h with 10^{-7} M TX and, after 1-h preincubation in fresh medium, treated over 3 h with either TX (TX+TX) or 10^{-8} M oestradiol-17β (E_2) (TX+E_2). Control groups consisted of anterior pituitary cells from 2-week OVX rats incubated for 75 h with medium alone (DMEM+DMEM) or 10^{-8} M E_2 (E_2+E_2). LH concentrations were determined during the 3 experimental hours of culture: basal (open bars), priming (filled bars) and primed (dashed bars). a, $P<0{\cdot}05$ vs basal hour. b, $P<0{\cdot}05$ vs priming hour. c, $P<0{\cdot}05$ vs primed hour in LHRH+LHRH group. d, $P<0{\cdot}05$ vs priming hour in LHRH+LHRH group; ANOVA followed by the Student–Newman–Keuls multiple range test.

In addition, they indicated that inhibitory action of E_2 on TX-induced LHRH self-priming was independent of the cAMP/PKA intracellular signal and oestrogen-dependent PR, and possibly related to deficient PR phosphorylation/activation by physiological specific phosphorylases. Unlike other SERMs (Sánchez-Criado *et al.* 2002, 2005*a*), TX is a type I oestrogen antagonist (Smith & O'Malley 2004), which, in the absence of oestrogen, displays agonistic effects on several tissues. At the rat pituitary level, these actions include: (1) stimulation of PRL secretion (Bellido *et al.* 2003, 2005, Aguilar *et al.* 2006); (2) shrinkage of hypertrophied gonadotropes due to OVX (Sánchez-Criado *et al.* 2004, 2006); (3) induction of PR mRNA (Bellido *et al.* 2003) and protein levels (Sánchez-Criado *et al.* 2006) in gonadotropes; and (4) LHRH self-priming without affecting the LHRH releasing pathway (Bellido *et al.* 2003).

In a TX environment, but not in an oestrogen background, E_2 blocked LHRH, P_4 and FSK potentiation of LHRH-stimulated LH secretion in both incubated pituitaries and cultured pituitary cells. This indicated that E_2 blocks both ligand (P_4)-dependent and ligand-independent (PKA) activation of PR. These novel findings are, additionally, supported by the lack of effect of E_2 in the increasing action of TX on gonadotrope PR expression or pituitary cAMP content. Like oestrogen, TX induces gonadotrope PR expression (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2005*a*, 2006, present results) and, in the absence of P_4, elicits LHRH self-priming. While the oestrogen-induced LHRH self-priming involves intracellular cAMP/PKA crosstalk with PR (Turgeon & Waring 1991, Waring & Turgeon 1992), the mechanism of TX-induced LHRH self-priming is not completely known. The involvement of PR is well documented, as oestrogen- and TX-induced LHRH self-priming can be suppressed (Sánchez-Criado *et al.* 2004) by blockade of PR action with P_4 antagonists RU486 or ZK299 (Beck *et al.* 1996). In fact, both LHRH self-priming and potentiation of LHRH-stimulated LH secretion by priming substances different from LHRH during the priming period (Pickering & Fink 1976) can be considered as physiological markers of PR activation in the gonadotrope.

TX was found to induce a significant and sustained increase in pituitary cAMP content, while the stimulating action of LHRH on cAMP content was absent. Previous reports point to an increasing effect of TX on the adenylyl cyclase system in the quail oviduct (Fanidi *et al.* 1992), in human mammary tumoral cells (Filardo *et al.* 2002) and in rat pituitary cells (Guelmes *et al.* 2005), but the mechanism and physiological meaning of this intriguing effect of TX is unknown. It is possible that increased cAMP production evoked by TX treatment in gonadotropes activates TX-induced PR in a ligand-independent manner, resulting in an increased LH response to LHRH (Bellido *et al.* 2003, present results). In contrast, oestrogen sensitizes the gonadotrope to the priming challenge of LHRH, giving rise to an ephemeral cAMP increase during the priming hour (Waring & Turgeon 1992, present results), which, in the absence of P_4, activates PR in a ligand-independent manner (Turgeon & Waring 1990, 1991, 1992, 1994). Therefore, the mechanisms by which E_2 and TX elicit LHRH self-priming seem to be different. In any case, no differences were found in gonadotrope PR expression or cAMP content after incubation of TX-treated pituitaries with E_2. These findings, together with those on the inhibitory action of E_2 on potentiated LHRH-stimulated LH secretion by LHRH, P_4, or FSK in TX-treated pituitaries or cells, indicate that the inhibitory action of E_2 on TX-induced LHRH self-priming was independent of the activation of PR by LHRH-independent cAMP/PKA pathway. Accordingly, an inefficient response of gonadotrope PR to physiological phosphorylation agents P_4 (Levine 1997) and PKA (Waring & Turgeon 1992) emerged as a possible explanation of the E_2 inhibition of TX-induced LHRH self-priming.

The results of the last experiment showed that inhibition of intracellular phosphatases with OA in cultured pituitary cells reversed the inhibitory action of E_2 on TX-elicited LHRH-stimulated LH secretion. Both P_4 (Denner *et al.* 1990, Takimoto & Horwitz 1993, McDonnell 1995) and LHRH-dependent cAMP/PKA intracellular signal (Zhang *et al.* 1994, Bai *et al.* 1997, Rowan *et al.* 2000) can phosphorylate the PR, a phosphoprotein transcription factor (Evans 1988, Mangelsdorf *et al.* 1995) that, upon phosphorylation, results in activation, transcription and regulation of gene expression (Beck *et al.* 1996). The absence of effect of P_4 and adenylyl cyclase activator FSK on TX-treated pituitaries or pituitary cells due to E_2 might well reveal a deficient basal or ligand-dependent phosphorylation response of PR (Takimoto & Horwitz 1993) to the physiological ligands P_4 or PKA (Rowan *et al.* 2000). PR phosphorylation is a complex ligand-dependent process (Denner *et al.* 1990, Takimoto & Horwitz 1993) that regulates steroid receptor function (Orti *et al.* 1992) and can be abolished by alkaline phosphatase treatment (Takimoto & Horwitz 1993). The present results showed that stimulation of protein phosphatases with CER reduced and blocked the E_2- and TX-induced LHRH self-priming respectively. Conversely, inhibition of protein phosphatases with OA, which is known to activate PR transcription in a dose-dependent manner (Denner *et al.* 1990), induced a similar LH response to LHRH regardless of the experimental incubation conditions of the present experiments. These findings indicate that the phosphoprotein PR of the gonadotrope was able to respond efficiently to phosphorylases, as shown by the increased LH response to LHRH. Accordingly, an imbalance in the intracellular kinase and phosphatase equilibrium (Zhang *et al.* 1994) due to membrane ER activation by E_2 (Sánchez-Criado *et al.* 2005*b*) would produce an alteration in the dynamic balance between PR phosphorylation and dephosphorylation rate, resulting in E_2-induced blockade of the TX-induced PR-dependent LHRH self-priming.

Since the only molecule that rats secrete with similar actions to TX is E_2 itself, results could be phenomenologic. However, our previous findings (Sánchez-Criado *et al.* 2005*b*) seem to indicate that, in the present model, TX is

acting as a genuine oestrogen agonist at the nuclear ER-α level in the gonadotrope. Taken together, these results indicate that the putative membrane ER-mediated E_2 inhibition of TX-induced LHRH self-priming in the rat (Sánchez-Criado *et al*. 2005*b*) is related to a blockade of hormone-dependent (P_4) and ligand-independent (PKA) activation of oestrogen-induced PR in gonadotropes, and that the lack of activation of PR is not due to a deficient response of the phosphoprotein PR to physiological phosphorylases, but to an imbalance of phosphorylase/phosphatase activities in the gonadotrope. The physiological significance of these findings could be that (i) the E_2-dependent LHRH self-priming compensates for the deficient spontaneous LHRH surge to trigger the normal LH surge (Fink 1995) and (ii) the moderate LH surge in the rat, about 10% of the maximal pituitary LH releasing capacity (de Koning *et al*. 2001), is a consequence of the integration of the inhibitory (non-genomic) and stimulatory (genomic) actions of oestrogen on PR-dependent LHRH self-priming.

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