Gonadotropin-secreting cells in ovariectomized rats treated with different oestrogen receptor ligands: a modulatory role for ERβ in the gonadotrope?

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Abstract

In the rat, oestrogen is a key regulator of gonadotrophin synthesis and release through activation of oestrogen receptors (ERs). Gonadotropes express α and β isoforms of ER and both can activate transcription in response to oestrogen. These experiments were aimed at evaluating the relative contribution of ERα and ERβ on gonadotrope morphology, progesterone receptor (PR) expression and LH secretion. Ovariectomized rats were daily injected over 3 days with 25 μg oestradiol benzoate, 0·3 or 1·5 mg of the selective ERα agonist propylpyrazole triol (PPT) with or without 1·5, 3·0 or 4·5 mg of the selective ERβ agonist diarylpropionitrile (DPN), DPN alone, and 0·3 or 3 mg of tamoxifen. Controls were given 0·2 ml oil. Serum concentration and pituitary content of LH, gonadotrope PR expression, pituitary PR content, and gonadotrope morphology were analyzed by RIA, immunohistochemistry, Western blotting and light and electron microscopy, respectively. Results showed that PPT reversed all consequences of ovariectomy, DPN mimicked the effects of PPT except for its LH-releasing action and tamoxifen had ERα-like responses. When combined with PPT, DPN attenuated ERα effects without interfering with its LH-releasing activity. Oestradiol benzoate had similar effects to those of combined PPT and DPN. It is suggested that (i) the structural reorganization of the cytoplasmic organelles provided by oestrogen, and the shrinkage of the ovariectomy-induced hypertrophy of gonadotropes, which precedes the expression of PR, are evoked by ERα and modulated, in a ying–yang fashion, by ERβ; and (ii) the oestrogen-dependent exocytosis of LH, the final step in the secretory process, is dependent on ERα exclusively.

Journal of Endocrinology (2006) **188,** 167–177

Introduction

In the rat, ovarian oestrogen regulates synthesis and release of gonadotrophins acting on intracellular oestrogen receptor (ER) in the gonadotrope. Synthesis and release of gonadotrophins are distinct and differentially regulated events. Whereas biosynthesis is a sophisticated process in the membrane-enclosed intracellular organelles – rough endoplasmic reticulum (RER) and Golgi complex – of the gonadotrope (Pierce 1988), release is a mere regulated exocytosis prompted by the physiological secretagogue gonadotrophin-releasing hormone (GnRH; Fink 1979, 1988). Removal of oestrogen by ovariectomy (OVX) in the rat induces profound effects on the synthesis and release of gonadotrophins. After OVX, pituitary content and release of gonadotrophins increase as time after OVX increases, and gonadotropes become hypertrophic with disorganization of intracellular organelles (OVX cells; Genbacev & Pantic 1975, Garner & Blake 1981). Also, as a consequence of the lack of oestrogen, the expression of progesterone receptor (PR) falls (Sánchez-Criado et al. 2004, 2005). Oestrogen treatment reverses the effects of OVX on gonadotrope size and morphology, PR expression and secretion of gonadotrophins (Genbacev & Pantic 1975, Low 1988, Sánchez-Criado et al. 2004).

Rat gonadotropes express ERα and ERβ isoforms (Mitchner et al. 1998, Vaillant et al. 2002, Sánchez-Criado et al. 2005). ER isoforms are encoded by separate genes and both can activate transcription in response to oestrogen (Kuiper et al. 1997, McInerney et al. 1998). After oestrogen binding to ERs, ER modulates the transcription of target genes and protein expression (Murdoch & Gorski 1991). This is followed at the pituitary level in ovariectomized rats by shrinkage of gonadotropes

and reorganization of the intracellular organelles, increase in gonadotrope responsiveness to the hypothalamic decapeptide GnRH and, through the activation of oestrogen-dependent PR (Turgeon & Waring 1994, Sánchez-Criado *et al.* 2005), GnRH self-priming (Fink 1995, 2000). By using the selective ERα and ERβ agonists, propylpyrazole trial (PPT) and diarylpropionitrile (DPN), respectively, in ovariectomized rats, we showed that selective activation of ERα restores the PR-dependent positive feedback of oestrogen on gonadotrophin release, sensitizes the pituitary to the secretagogue GnRH, and induces GnRH self-priming (Sánchez-Criado *et al.* 2004). In addition to these effects on gonadotrophin release, ERα induces PR mRNA and protein expression and reduces the number and size of OVX cells. In contrast, selective activation of ERβ lacks releasing effects while it induces PR expression, not followed by GnRH self-priming, and reduces the number and size of OVX cells (Sánchez-Criado *et al.* 2004).

The existence of steroid receptor subtypes is becoming more the rule than the exception, and there is increasing evidence to suggest that the presence of receptor isoforms is not a case of redundancy but rather the indication of an additional level of regulation at the target tissue (Keightley 1998). The aim of the present study was to evaluate, in ovariectomized rats, the effect of different ER ligands (the cognate ligand oestradiol benzoate, selective ERα and/or ERβ agonists and the selective ER modulator tamoxifen (TX)) on: (i) light and electron microscopy of the gonadotrope morphology; (ii) PR protein expression and content in the gonadotrope and the anterior pituitary, respectively; and (iii) serum concentration and pituitary content of luteinizing hormone (LH).

Materials and Methods

Animals and treatments

Adult female Wistar rats weighing 190–210 g were used. Rats were housed under a 14 h:10 h light/dark cycle (light on at 05:00 h) and 22 ± 2 °C room temperature, with access *ad libitum* to rat chow and tap water. All rats were ovariectomized under light ether anesthesia at random stages of the oestrous cycle. Two weeks after surgery, ovariectomized rats were injected daily at 09:00 h over 3 days with (i) 0·2 ml oil vehicle (control group), (ii) 25 μg of oestradiol benzoate (EB; Sigma Chemical Co.) to obtain maximal oestrogen effects, (iii) 0·3 or 1·5 mg of the ERα-subtype-selective ligand PPT (Tocris Cookson, Avonmouth, Avon, UK; Stauffer *et al.* 2000) with or without 1·5 mg of the ERβ-subtype-selective ligand DPN (Tocris Cookson; Meyers *et al.* 2001), (iv) DPN alone, or (v) 0·3 or 3 mg of the selective ER modulator TX (Sigma Chemical Co.). Doses of ER-selective ligands used were chosen based on previous studies showing that

PPT and DPN displayed full oestrogen activity and absence of ERα activation, respectively (Sánchez-Criado *et al.* 2004, Tena-Sempere *et al.* 2004). At 09:00 h on the day after treatments rats were decapitated, their trunk blood collected and serum stored at − 20 °C until quantified for LH by RIA. The anterior pituitaries were dissected out and processed for (i) pituitary LH content determination, (ii) immunohistochemistry and electron microscopy studies, and (iii) Western blot analysis. Additional ovariectomized rats treated with 1·5 mg PPT were injected with 0·2 ml oil or 1·5, 3·0, or 4·5 mg DPN and their pituitaries evaluated for immunohistochemistry. All experimental protocols were approved by the Ethical Committe of the University of Córdoba. Experiments were performed in accordance with the rules of laboratory animal care and international law on animal experimentation.

Pituitary LH content determination

For LH content determination, anterior pituitaries were homogenized in 1 ml RIA buffer and subjected to ultrasonic treatment. Samples were centrifuged at 2800 g for 10 min and the supernatants frozen at −20 °C until assayed by RIA.

RIA of LH

Serum concentration and pituitary content of LH were measured in duplicate by RIA using a double-antibody method with kits supplied by the National Institutes of Health (Bethesda, MD, USA) and a previously described microassay method (Sánchez-Criado *et al.* 1990). Rat LH-I-10 was labelled with ^{125}I by the chloramine T method. All serum and pituitary samples were assayed in the same assay. The intra-assay coeffient of variation was 8%. Assay sensitivity was 3·75 pg/tube. LH concentration and content were expressed as ng/ml serum or μg/mg pituitary of the reference preparation LH-rat-RP-3.

Tissue preparation for light and electron microscopy

Anterior pituitaries from four rats/group were divided into half. For light microscopy, four hemipituitaries/group were fixed in 10% buffered formalin and embedded in paraffin wax. For electron microscopy, small fragments from each of the remaining four hemipituitaries/group were fixed in 2·5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) followed by post-fixation in 1% osmium tetroxide in distilled water for 2 h. Tissue fragments were then embedded in Araldite and cut at 0·1 μm with an ultramicrotome. Current functional ultrastructural characteristics of membrane-enclosed intracellular organelles (RER, Golgi complex, and secretory vesicles) in gonadotrophin-secreting cells (Childs 1986) were evaluated.

Immunohistochemistry of pituitary PR

The immunohistochemical study was performed on dewaxed and rehydrated 3 μm thick tissue sections of formalin-fixed, paraffin-embedded tissue samples. The commercial mouse monoclonal anti-human PR antibody clone PR10A9, which is raised against the recombinant hormone-binding domain in the C-terminal region of human PR (Immunotech, Marseille, France; diluted 1:15 000), and the avidin–biotin peroxidase complex technique (Vector Laboratories, Burlingame, CA, USA), were used as previously described (Sánchez-Criado *et al.* 2004). 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 lack of staining after pre-incubation of tissue sections of rat uterus and pituitaries from ovariectomized rats treated with EB with 10^{-9}, 10^{-7} and 10^{-5} M of the cognate ligand 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 a negative control. Several dilutions of the PR10A9 monoclonal antibody were tested and the optimal dilution was established at 1:15 000, because it gave the highest intensity of nuclear staining with the lowest background staining in pituitary and uterus (Sánchez-Criado *et al.* 2004). Nuclear counter-staining was performed with Mayer's haematoxylin in all cases.

Identification of gonadotropes and morphometric study

All pituitary cells expressing PR were identified as activated gonadotropes because gonadotropes are the only pituitary cells expressing PR (Fox *et al.* 1990). In addition, gonadotropes not expressing PR were identified because they had the size and morphology features of OVX cells (hypertrophied gonadotropes; Garner & Blake 1981, Childs 1986). The size of pituitary cells (their perimeter) was evaluated in digitalized images of PR–immunostained tissue sections using the VISILOG 5 program as described previously (Blanco *et al.* 2001). Twenty digitalized fields per hemipituitary were evaluated. Each field was digitalized at $400\times$ magnification.

Protein extraction and Western blotting for pituitary PR content

Relative levels of PR protein were assessed by Western blot using a commercial polyclonal antibody (PR C-20: sc-539; Santa Cruz Biotechnology), and previously published protocols (Guerra-Araiza *et al.* 2003), with minor modifications. Briefly, pituitaries (four/group) were collected immediately upon decapitation, snap-frozen and stored at -80 °C. Tissue fragments were homogenized in TDG lysis buffer containing 10 mM Tris/HCl,

1 nM dithiothreitol, 30% glycerol, 1% Triton X-100, 15 mM sodium azide and 1 mM EDTA, supplemented with the protease inhibitor mix CLAP (5 mg/ml chymostatin, 5 mg/ml leupeptin, 5 mg/ml antipain and 5 mg/ml pepstatin-A) and 1 mM PMSF. After protein quantification using the Bradford method, equal amounts of proteins (150 μg/lane) were resolved by electrophoresis in 10% SDS/PAGE gels at 75 V. Gels were transferred to nitrocellulose membranes (Amersham Biosciences) overnight at 30 V. After transfer, membranes were blocked for 2 h in 5% non-fat dry milk in Tris-buffered saline, and incubated overnight at 4 °C in the presence of 2 μg/ml primary anti-PR antibody. After exhaustive washing, membranes were incubated for 1 h with goat anti-rabbit IgG conjugated with horseradish peroxidase. Proteins were detected by chemiluminescence, using the ECL kit (Amersham Biosciences) following manufacturer's instructions.

As a control for the specificity of PR immunodetection, reactions with the primary antibody omitted were routinely conducted. In addition, as an internal control for the amount of protein loaded per lane, membranes were reprobed with a goat anti-β-actin polyclonal antibody (Actin C-11: sc-1615; Santa Cruz Biotechnology), as desribed above. Quantification of the intensity of Western blot signals was carried out by densitometric scanning using an image-analysis system (1-D Manager; TDI, Madrid, Spain), and values for the specific signal (PR) were normalized to those of the internal control (β-actin) to express arbitrary units of relative expression.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) to test for significant differences among groups. When significant differences existed, ANOVA was followed by the Student–Newman–Keuls multiple range test to compare means. The level of significance was taken to be 0·05 level.

Results

Effect of ER ligands on serum and pituitary LH levels in ovariectomized rats

Administration of EB, 1·5 mg PPT, with or without DPN, and 3 mg TX decreased serum LH concentrations in ovariectomized rats. The remaining treatments had no effect (Fig. 1, upper panel). Pituitary LH content in ovariectomized rats decreased significantly after treatment with EB, 1·5 mg PPT, and 3 mg TX only. The decreasing effect of 1·5 mg PPT on LH pituitary content was blunted by the simultaneous administration of DPN (Fig. 1, lower panel).
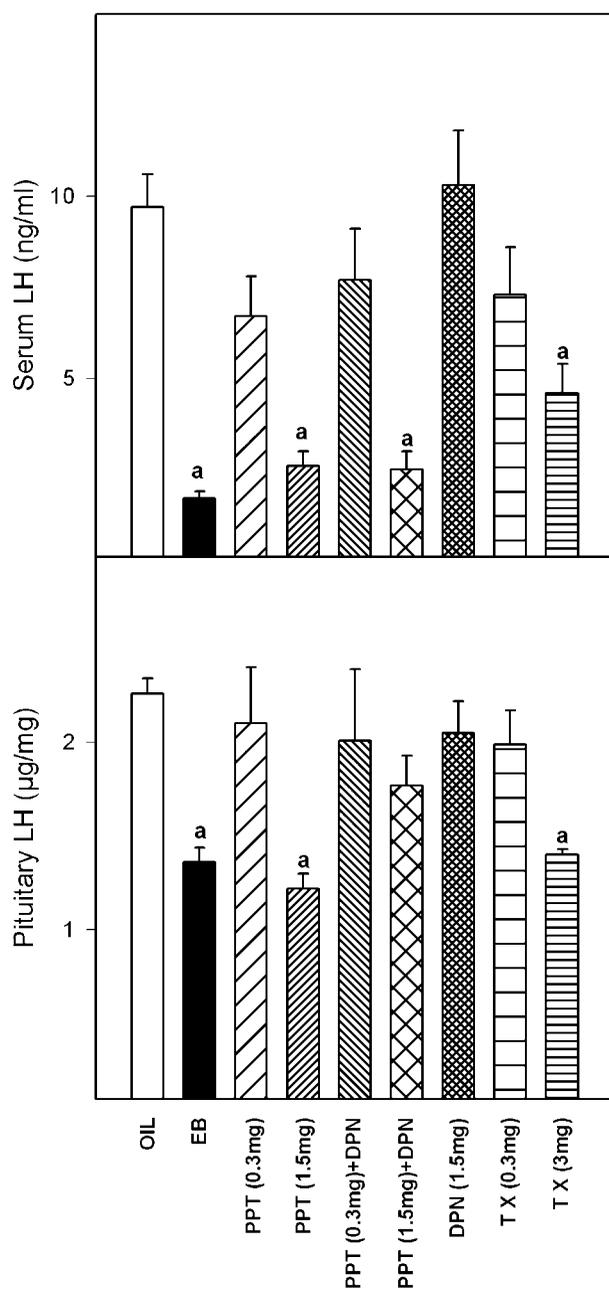
Figure 1 Serum concentration ($n=14$) and pituitary content ($n=5$) of LH in 2-week ovariectomized rats given 0·2 ml oil, 25 µg EB, 0·3 or 1·5 mg PPT with or without 1·5 mg DPN, 1·5 mg of DPN alone, or 0·3 or 3 mg of TX over 3 days. [a]$P<0·05$ versus ovariectomized rats injected with oil.

Structural and ultrastructural features of gonadotropes in ovariectomized rats treated with ER ligands

The percentage of gonadotropes found in this study ($14·1 \pm 0·2$ of the total pituitary cells), which was in agreement with that reported in gonadectomized rats

(Childs *et al*. 1982, Smith & Keefer 1982), was not affected by any ER ligand treatments. On the contrary, differences in the size of gonadotropes were noted between control and experimental groups, although the sizes of other pituitary cells was not compromised (see legends of Figs 3 and 7, below). Thus, pituitaries of the control group (ovariectomized rats injected with oil) had hypertrophied but not activated gonadotropes, while those of ovari-ectomized rats treated with ER ligands displayed both hypertrophied and activated gonadotropes (Figs. 2A and 3).

Structurally, hypertrophied gonadotropes were large, rounded cells with foamy or, more often, vacuolated cytoplasms and central or eccentric nuclei (Fig. 2A). Ultrastructurally, hypertrophied gonadotropes had dilated cisternae of RER, forming large vesicles, with irregular enlarged and prominent Golgi complexes. Eventually, cells with a single dilated RER vesicle with a homogeneous content of low electron density were observed (signet ring cells; Fig. 4A). Both the mean perimeter and the number of hypertrophied gonadotropes from ovari-ectomized rats treated with ER ligands were always smaller than those of hypertrophied gonadotropes from the control group of ovariectomized rats (Fig. 3). Thus, the mean perimeter of hypertrophied gonadotropes of the control group was $68·3 \pm 0·3$ µm, which sharply con-trasted with the mean perimeter of the other pituitary cells ($38·1 \pm 2·9$ µm; see legend of Fig. 3), while the mean perimeter of hypertrophied gonadotropes from the experimental groups ranged from 65·3 µm in OVX+TX (0·3 mg) to 61·9 µm in OVX+PPT (1·5 mg)-treated rats. Also, a decrease in the percentage of hypertrophied gonadotropes was noted in the experimetal groups: 4·4% in OVX+PPT (1·5 mg)-treated rats to 11·1% in OVX+ TX (0·3 mg)-treated rats with respect to ovariectomized+ oil-injected rats (14·1%).

Activated gonadotropes were characterized by the presence of PR at the nuclear level (Fig. 2B and C) and were exclusively found in the pituitaries of ER-ligand-treated rats. The number of activated gonadotropes and the intensity of staining were highest in rats injected with 1·5 mg PPT (Figs 2B and 3) and lowest in rats injected with 0·3 mg TX (Figs 2C and 3). The structural and ultrastuctural features of activated gonadotropes also varied and, overall, electron microscopy changes in RER, Golgi complex, and secretory vesicles in the gonadotrope in ovariectomized rats treated with ER ligands corre-lated well with those observed at light microscopic level (Figs. 2, 4, and 5). Structurally activated gonadotropes were round, ovoid, and polygonal cells, with vacuolated, foamy, or homogeneous cytoplasms and eccentric or central nuclei (Fig. 2B). Electron microscopy disclosed dilated RER cisternae forming from rounded (Figs 4B and C) to ovoid (Fig. 4D) and polygonal (Figs 4E and F) vesicles. Golgi complexes stood out more prominently in activated gonadotropes with ovoid and irregular vesicles

(Fig. 4D) than in those with rounded, homogeneous vesicles (Figs 4B and C), while no differences in granulation were apparent. The perimeter of activated gonadotropes was always smaller than the perimeter of hypertrophied gonadotropes in any group of ER–ligand-treated rats. The perimeter threshold for the change
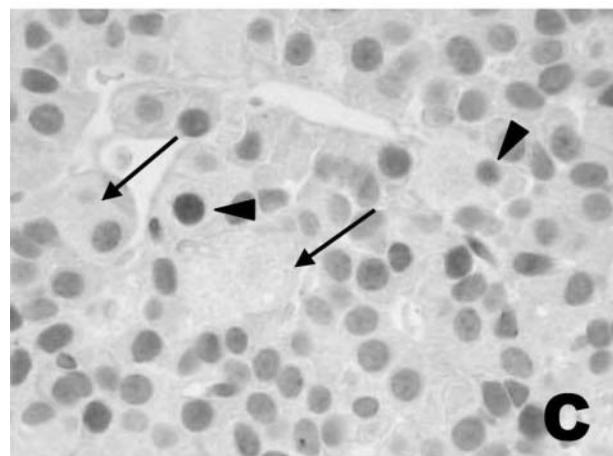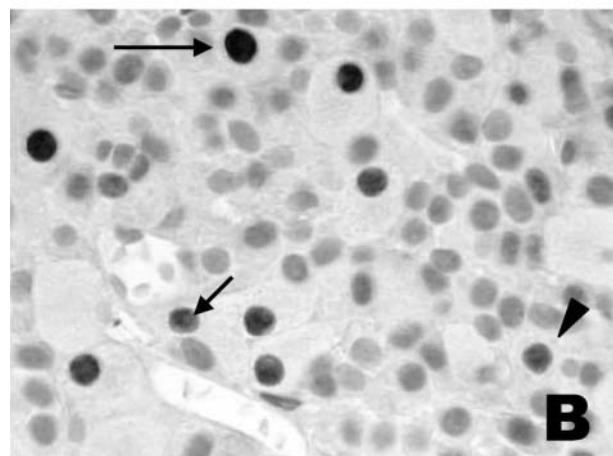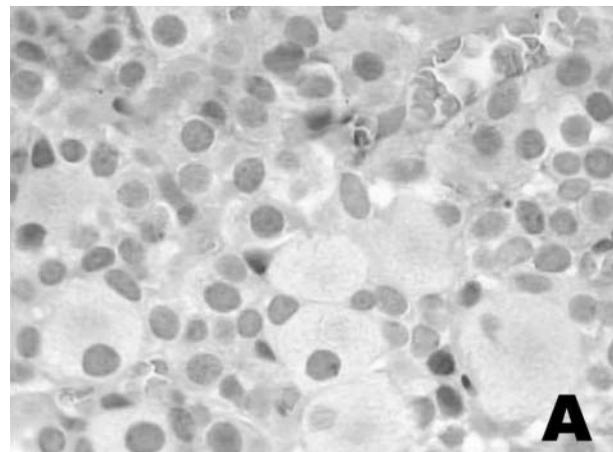
from hypertrophied to activated gonadotrope appeared to lie between 50 and 60 μm. The relative number of activated gonadotropes ranged from 3·7% in OVX+TX (0·3 mg)-treated rats to 10·7% in OVX+PPT (1·5 mg)-treated rats (Fig. 3). Both hypertrophied and activated gonadotropes from the OVX+EB-treated group and the OVX+PPT (1·5 mg)+DPN–treated group of rats were very similar in terms of percentage and size (Fig. 3), as well as structural and ultrastuctural features (Figs 4B and C). It is interesting to highlight that the administration of DPN to rats injected with 0·3 or 1·5 mg PPT significantly blunted the oestrogen effects of PPT on the gonadotrope, both at the structural (Fig. 3) and ultrastructural (Fig. 5) levels.

Effect of ER ligands on PR protein expression in ovariectomized rats

Western blot analysis of PR protein in the pituitary revealed an intense band of approximately 115 kDa, apparently corresponding to the B isoform of PR (PR–B), in keeping with previous studies (Guerra-Araiza *et al.* 2003; Fig. 6, upper panel). Semi-quantitative evaluation of the expression data showed a low to negligible PR–B signal in ovariectomized rats. In this model, administration of EB induced a significant increase in PR protein content, a response that was mimicked by administration of PPT at the doses of 0·3 and 1·5 mg. Likewise, DPN induced a modest but significant increase in PR–B protein levels; yet this response was significantly lower than that induced by PPT. Co-administration of PPT and DPN at doses of 1·5 mg resulted in changes in PR–B levels over OVX values that were lower than those induced by administration of PPT alone (Fig. 6, lower panel). Finally, treatment with TX dose-dependently enhanced PR–B protein levels, as 0·3 mg TX was ineffective, whereas 3 mg TX increased PR–B content. However, the magnitude of the response to the highest dose of TX was

Figure 2 Structural features and PR-expression patterns of gonadotropes from 2-week ovariectomized rats injected over 3 days with different ER ligands (see legend of Fig. 1 for details of treatments). Avidin–biotin peroxidase complex immunohistochemical technique, counterstaining with Mayer's haematoxylin, × 400. (A) In ovariectomized rats injected with oil, the large, rounded cells with clear and vacuolated cytoplasm are hypertrophied gonadotropes. Nuclear staining with PR antibody is absent. (B) In ovariectomized rats treated with PTT (1·5 mg), immunoreactive products to PR antibody are seen in the nuclei of some cells (dark colour) which are thus identified as activated gonadotropes. The intensity of staining varies from high (long arrow) to medium (short arrow). Some activated gonadotropes are rounded with clear cytoplasm and some others are smaller and polygonal (arrowhead). (C) In ovariectomized rats treated with TX (0·3 mg), large, rounded cells with clear, occasionally vacuolated cytoplasm are seen (arrows). Two of them show a faint nuclear staining (arrowheads).
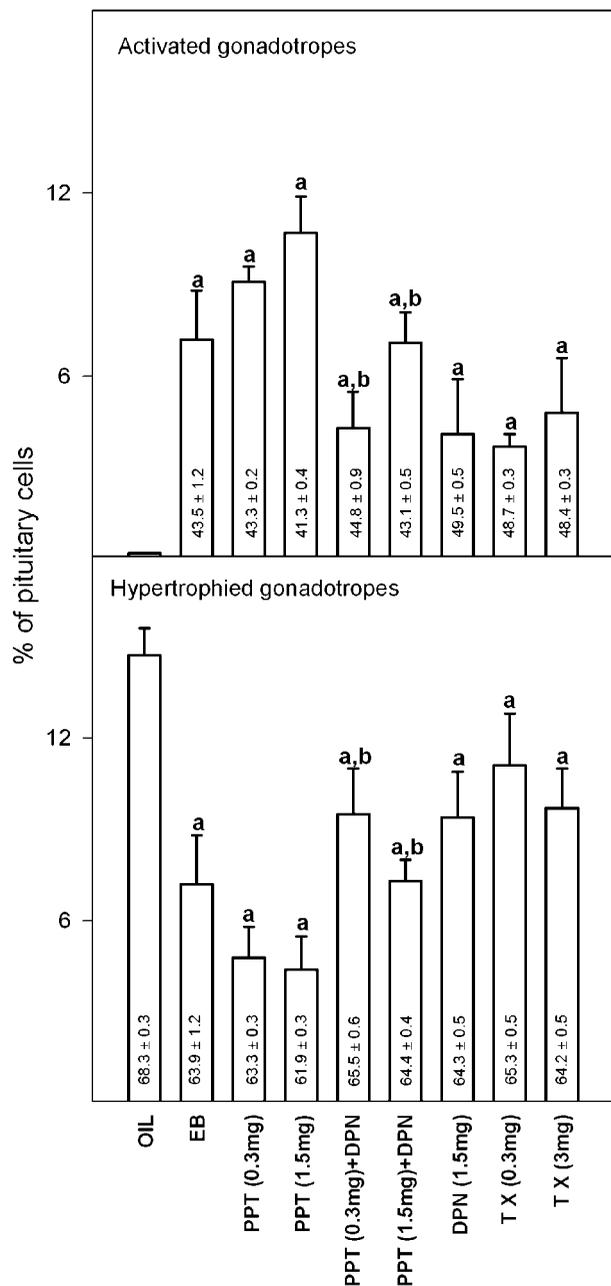
Figure 3 Relative number (%) of pituitary cells showing (activated gonadotropes) or not showing (hypertrophied gonadotropes) immunohistochemical reaction to PR in pituitaries from 2-week ovariectomized rats injected over 3 days with different ER ligands (see legend of Fig. 1 for details of treatments). Values are means ± S.E.M. from four hemipituitaries/group (20 fields of about 15 gonadotropes/rat). [a]$P<0.05$ versus ovariectomized rats injected with oil; [b]$P<0.05$ versus values in pituitaries from ovariectomized rats injected only with 0.3 or 1.5 mg PPT. Inside each bar is the mean ± S.E.M. (μm) of the gonadotrope perimeter. The average perimeter of the remaining pituitary cells (85.7 ± 4.0%) was 38.1 ± 2.9 μm and was not affected by ER ligand treatments.
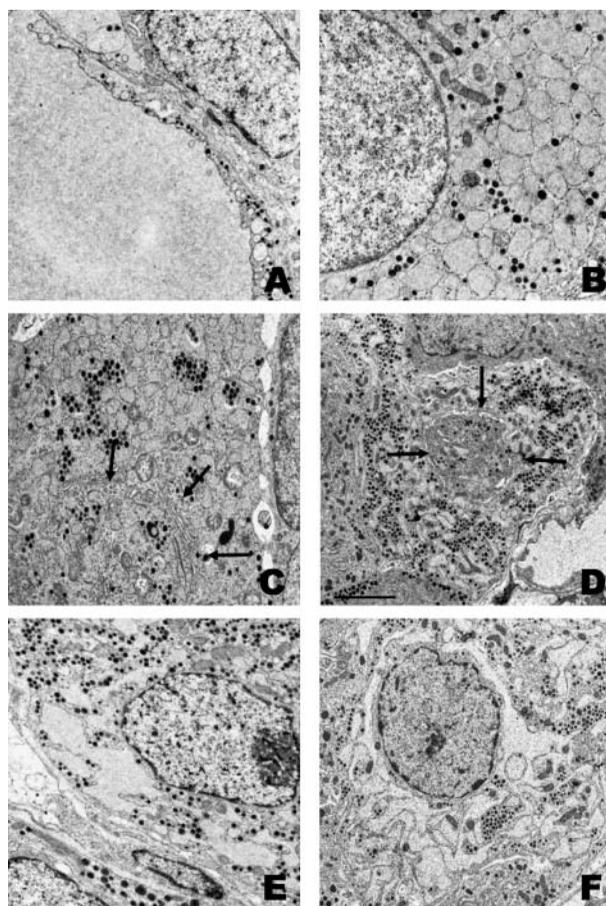

Figure 4 Ultrastructural features of activated gonadotropes from 2-week ovariectomized rats injected over 3 days with different ER ligands (see legend of Fig. 1 for details of treatments). (A) Signet ring cell in an ovariectomized rat injected with oil. A single, large RER vesicle of homogeneous, low electron density content is seen, × 15 000. (B) Gonadotrope with a vesiculated cytoplasm in an ovariectomized rat treated with EB. The vesicles are small, rounded and uniform in size and have a low electron density content. The nucleus has a rounded outline and secretory granules are scattered throughout the cytoplasm, × 15 000. (C) Gonadotrope in an ovariectomized rat injected with PPT (1.5 mg) with a vesiculated cytoplasm similar to that shown in (B) and a visible Golgi complex (arrows). Secretory granules are scattered throughout the cytoplasm, × 4000. (D) Gonadotrope in an ovariectomized rat injected with PPT (1.5 mg) and DPN (1.5 mg). The cytoplasm is vesiculated but vesicles are ovoid rather than rounded and their size is not homogeneous. It is also well granulated and an enlarged Golgi complex is seen (arrows), × 4000. (E) Gonadotrope in an ovariectomized rat injected with DPN (1.5 mg) with large, irregular vesicles of different sizes with few tenous strands of cytoplasm and granules between them. The nucleus is heterochromatic and has a large nucleolus, × 4000. (F) Gonadotrope of an ovariectomized rat injected with 3 mg TX with dilated cisternae of RER forming a filigree in the cytoplasm, which is well granulated, × 4000.
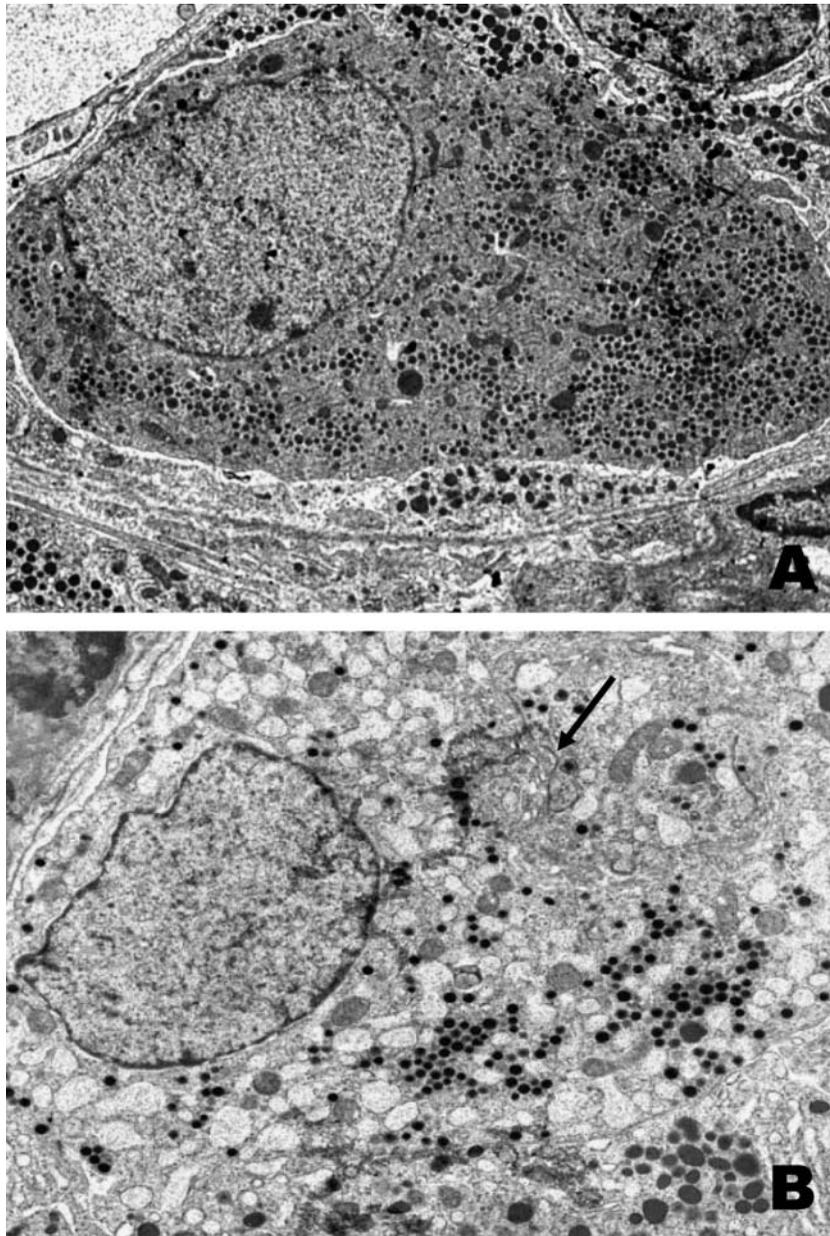
Figure 5 Micrographs showing comparative details of the effect of 1·5 mg DPN on the ultrastuctural features of gonadotropes from ovariectomized rats treated with 1·5 mg PPT (see legend of Fig. 1 for details of treatments). (A) Gonadotrope from an ovariectomized rat treated with PPT alone with a polygonal shape, an oval, euchromatic nucleus and a homogeneous cytoplasm. Secretory granules are scattered throughout the cytoplasm, × 7600. (B) Gonadrotrope from an ovariectomized rat after the combined treatment with PPT and DPN shows an increase in cell size, a slightly irregular nucleus and a vesiculated cytoplasm. The vesicles are small, ovoid and nearly uniform in size and they virtually fill the cytoplasm. Granules are scattered between the vesicles throughout the cytoplasm and vesicles and cisternae of the Golgi complex are seen (arrow), × 7600.
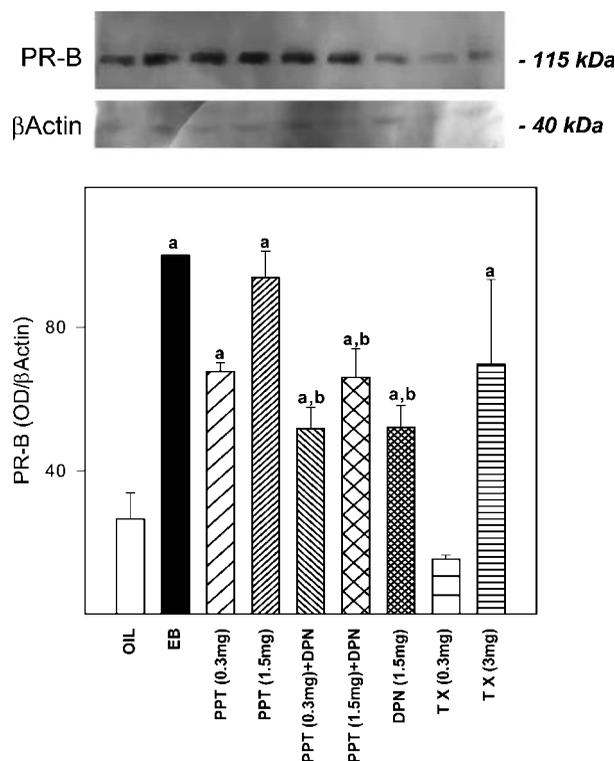
Figure 6 PR protein content in pituitary samples from 2-week ovariectomized rats injected over 3 days with different experimental ER ligands (see legend of Fig. 1 for details of treatments). In the upper panel, representative Western blots of PR and β-actin are shown. Probing of blots with a specific anti-PR polyclonal antibody evidenced a single band of approximately 115 kDa, which corresponds to the predicted size of the PR-B isoform. Thereafter, blots were stripped and reprobed with β-actin antibody; a procedure which resulted in detection of a single band of the predicted size 40 kDa. The lower panel shows a compilation of semi-quantitative data of relative PR-B protein levels in the experimental samples. Data are means ± S.E.M. from five independent Western blot assays. [a]$P<0.05$ versus ovariectomized rats injected with oil; [b]$P<0.05$ versus ovariectomized rats injected with 1·5 mg PPT.
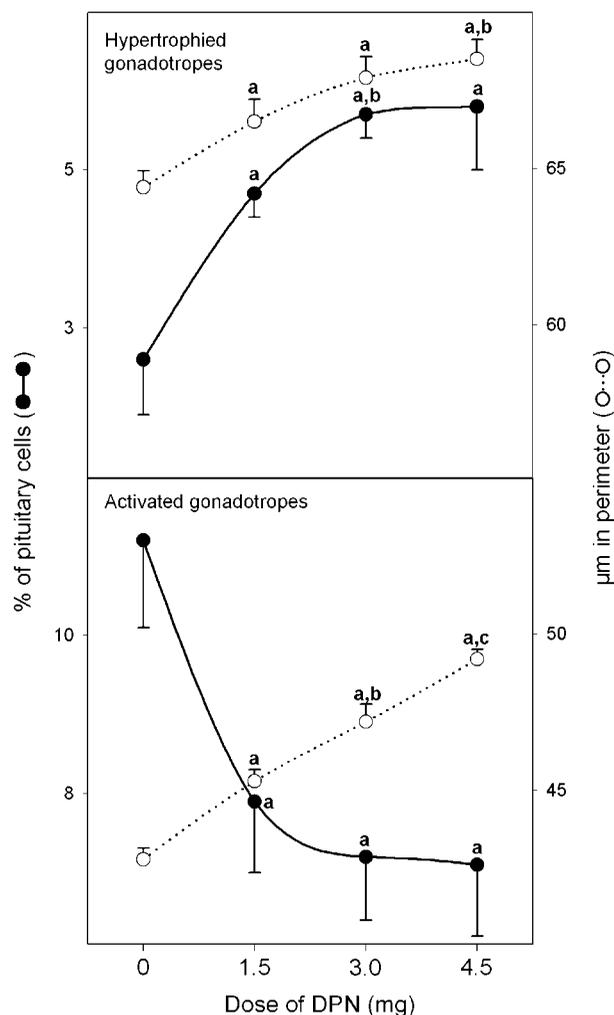

Figure 7 Relative number (%) and average perimeter (μm) of pituitary cells showing activated (lower panel) and hypertrophied (upper panel) gonadotropes in pituitaries from 2-week ovariectomized rats injected over 3 days with 1·5 mg PPT and with 0·2 ml oil, 1·5, 3·0, or 4·5 mg DPN. Values are means ± S.E.M. from four rats (20 fields of about 15 gonadotropes/rat). [a]$P<0.05$ versus ovariectomized+PPT rats injected with oil; [b]$P<0.05$ versus ovariectomized+PPT rats injected with 1·5 mg DPN; [c]$P<0.05$ versus ovariectomized+PPT rats injected with 3 mg DPN. The average perimeter of the remaining pituitary cells (86·3 + 8·0%) was 39·9 + 4·9 μm, and was not affected by ER ligand treatments.

significantly lower than that induced by 1·5 mg PPT (Fig. 6, lower panel).

Effect of different doses of DPN on gonadotropes in ovariectomized rats treated with 1·5 mg PPT

Figure 7 depicts the effect of increasing doses of DPN on the percentage and perimeter of activated and hypertrophied gonadotropes in ovariectomized rats injected over 3 days with 1·5 mg PPT. Whereas the percentage of activated gonadotropes decreased, that of hypertrophied gonadotropes increased in a DPN dose–dependent manner. Also, DPN blunted, in a dose–dependent manner, the gonadotrope shrinkage induced by PPT in ovariectomized rats.

Discussion

The results of the present work showed that administration of ERα-subtype-selective ligand PPT (Stauffer *et al.* 2000) in ovariectomized rats had oestrogen activity on all parameters of gonadotrope function analysed, whereas administration of ERβ-subtype-selective ligand DPN (Meyers *et al.* 2001) mimicked all actions of PPT except the reduction of pituitary content and serum

concentration of LH. The latter action, however, was the only one not affected when both ER ligands were administered simultaneously. Morphological parameters of gonadotrope function evaluated were qualitative characteristics of the membrane-enclosed intracellular organelles, gonadotrope size, and PR expression status. In fact, due to the pivotal role of PR in gonadotrope function in the rat (Fink 2000), we used the immuno-histochemical expression of PR in gonadotropes to classify them as activated (with PR expression) or hypertrophied (without PR expression).

In the rat, the presence of PR in gonadotropes is an absolute requirement for full pituitary responsiveness to GnRH (Fink 1995, 2000). Strictly oestrogen-dependent PR (Fox *et al*. 1990) can be activated by the cognate ligand (Levine 1997) or, in a ligand-independent manner, by intracellular signalling of GnRH (Turgeon & Waring 1994). In the absence of PR expression (Chappell *et al*. 1999, Bellido *et al*. 2003) or action (Sánchez-Criado *et al*. 2004) GnRH self-priming is absent; hence, the height of the LH surge and the ovulation rate are reduced (Rao & Mahesh 1986, Sánchez-Criado *et al*. 1990, Chappell *et al*. 1999). A close inverse relationship was noted between gonadotrope size and PR expression. Gonadotropes below 50 μm in perimeter always expressed PR (activated), while those greater than 60 μm did not (hypertrophied). Thus, a clear cut-off of about 55 μm in perimeter seemed to be the threshold for acquisition of the ability to express PR (Fig. 7) and, in consequence, full responsiveness to GnRH. Classification of gonadotropes on a purely morphological basis, regardless of whether they express PR or not (Sánchez-Criado *et al*. 2004), gives overlapping figures and may mask ERβ effects. The present experiments showed that the percentage of activated gonadotropes paralleled the pituitary content of PR protein in the different experimental groups, as gonadotropes are the only pituitary cell type expressing PR (Fox *et al*. 1990, Sánchez-Criado *et al*. 2005). Minor differences noted in PR levels determined by immunohistochemistry and Western blot, qualitative and semi-quantitative techniques, respectively, could be due to the higher sensitivity of immunohistochemistry, as revealed in 0·3 mg TX-treated ovariectomized rats (Figs 2C and 3), and the heterogeneous distribution (Fig. 2B) of the same amount of PR protein among gonadotropes. Overall, PR protein levels were low in ovariectomized rats, high in ovariectomized rats injected with EB or PPT, and intermediate in ovariectomized rats given DPN or TX, as already described for PR mRNA (Bellido *et al*. 2003). Finally, and much more important, activation of ERβ with DPN reduced the effect of the potency-selective ERα agonist PPT on the number of gonadotropes expressing PR and pituitary content of PR protein in ovariectomized rats. In addition to these effects on PR expression, the results of the present study showed that activation of ERα with PPT reversed the effects of OVX on gonadotrope

morphology and serum levels and pituitary content of LH. Activation of ERβ with DPN had only slight effects on gonadotrope morphological parameters, with no evidence of negative feedback on LH release. Concurrent activation of both ER isoforms by simultaneous administration of PPT and DPN had intermediate shrinking and morphological effects on hypertrophied gonadotropes and on pituitary LH content without affecting the negative-feedback effect of PPT on LH release.

Activation of ERα in ovariectomized rats seemed to be responsible for all actions of oestrogen in the gonadotrope: (i) negative feedback on LH release; (ii) induction of PR; and (iii) reorganization of the membrane-enclosed intracellular organelles and shrinkage of gonadotropes. Moreover, it has previously been shown that PPT induces in ovariectomized rats: (i) positive feedback on LH release, (ii) sensitization of gonadotropes to GnRH, and (iii) PR-dependent (Chappell *et al*. 1999) GnRH self-priming (Sánchez-Criado *et al*. 2004). The results obtained here with TX, a selective oestrogen receptor modulator (SERM) that displays antagonist/agonist actions (McDonnell 1999, McDonnell *et al*. 2002), supported this role for ERα. In the absence of the cognate ligand, TX induces PR expression and elicits GnRH self-priming (Bellido *et al*. 2003, Sánchez-Criado *et al*. 2005), most probably through the ERα isoform (Tzuckerman *et al*. 1994, Sánchez-Criado *et al*. 2005). The present results showed that 3 mg TX, but not 0·3 mg, significantly reduced serum and pituitary levels of LH in ovariectomized rats, an obvious ERα effect, and reverted, in a dose-related manner, the effects of OVX on gonadotrope hypertrophy and PR expression. On the other hand, activation of ERβ alone lacked both positive (Sánchez-Criado *et al*. 2004) and negative feedback effect on LH release (present results). In contrast with the lack of effect on pituitary content and serum concentration of LH, ERβ induced, in the absence of ERα, reorganization of the membrane-enclosed intracellular organelles (RER and Golgi complex) and shrinkage of gonadotropes.

Potentially more important were the findings of the simultaneous administration of PPT and DPN. These results demonstrated, for the first time, that activation of ERβ with DPN reduced, in a dose-related manner, the shrinking effect of ERα activation on gonadotropes and on ERα-stimulated PR levels. Also, DPN blunted the decreasing effect of PPT on pituitary LH content without influencing PPT effect on serum LH concentration. It is worth noting that daily administration of 25 μg EB, the cognate ligand that activates both ER isoforms (McInerney *et al*. 1998, Hall & McDonnell 1999) over 3 days, had a full suppressive effect on LH release in ovariectomized rats, whereas its effects on gonadotrope morphology, the number of gonadotropes expressing PR, and pituitary LH content were moderate and similar to those elicited by coactivation of ERα and ERβ with their selective agonists. The pharmacological doses of EB used in

the present experiment shrinks gonadotropes almost to the size exihibited by pituitaries in intact, proestrous rat (Sánchez-Criado *et al.* 2005). The respective roles of ERα and ERβ in prolactin- and gonadotrophin-secreting cells have previously been investigated through the use of ER-knockout mice (Pelletier *et al.* 2003). However, the lack of either ERα or ERβ in ER-knockout models limits the study of physiological interaction between ER isoforms.

The majority of steroid receptors (glucocorticoid, mineral corticoid, androgen, progesterone and oestrogen) come in pairs, and evidence is accumulating to indicate that this is not a case of redundancy, but that it has a physiological modulatory meaning (Vegeto *et al.* 1993, Oakley *et al.* 1996, Paech *et al.* 1997, Hall & McDonnell 1999, Conneely *et al.* 2001). In many contexts, ERβ appears to antagonize the actions of ERα in a ying–yang relationship (Paech *et al.* 1997, Hall & McDonnell 1999, McDonnell *et al.* 2002, Weihua *et al.* 2003, Koehler *et al.* 2005). ERβ inhibits the stimulatory action of ERα on breast (Paruthiyil *et al.* 2004, Helguero *et al.* 2005), bone (Lindberg *et al.* 2003), uterus (Weihua *et al.* 2000, Frasor *et al.* 2003), and hair follicles (Ohnemus *et al.* 2005) in mice. The present results showed a similar relationship between ERα and ERβ in the ovariectomized rat gonadotrope. In the absence of activation of ERα, which predominates in the pituitary (Scully *et al.* 1997), activation of ERβ partially replaced its actions on gonadotrope shrinkage, reorganization of intracellular organelles, and PR expression. In the presence of activated ERα, ERβ reduced the effects of ERα on gonadotrope morphology, PR expression, and pituitary LH content. On the contrary, activation of ERβ, either alone or in combination with ERα, did not prompt an inhibitory effect on serum LH concentration in ovariectomized rats. In addition, it has previously been shown that ERβ, alone or combined with ERα, has no effects on basal or GnRH-stimulated LH release nor on GnRH self-priming (Sánchez-Criado *et al.* 2004) despite its effects on PR. All these results implied that release and the rest of the secretory events in the gonadotrope are differentially ER-regulated events.

In summary, reorganization of the membrane-enclosed intracellular organelles, shrinkage of the gonadotrope, PR expression, and reduction of pituitary LH content in ovariectomized rats are positively regulated by ERα, and these actions of ERα are inhibited by ERβ. In the absence of ERα activity, activation of ERβ partially replaced these actions. Regulated exocytosis, the final process in the secretory pathway, is dependent exclusively on ERα priming. Thus, it appeared that the presence of ERα and ERβ in the gonadotrope is not a case of redundant regulatory effects of oestrogen, but rather the results suggested the existence of a more refined level of modulation of oestrogen signalling at the level of the gonadotrope.

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