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Estradiol and its membrane-impermeable conjugate estradiol–BSA inhibit tamoxifen-stimulated prolactin secretion in incubated rat pituitaries

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Abstract

In the absence of estrogen (E), the selective E receptor modulator tamoxifen (TX) has two agonist effects in the rat pituitary: induction of progesterone receptor (PR)-dependent GnRH self-priming in the gonadotrope, and stimulation of prolactin (PRL) secretion in the lactotrope. TX-induced gonadotropin (GnRH) self-priming is absent when 10^{-8} M estradiol-17β (E_2) is added to the incubation medium of pituitaries from TX-treated rats. The present experiments investigated whether PR-independent PRL release into the incubation medium of pituitaries from TX-treated ovariectomized (OVX) rats was affected by E_2, and the effect of different ER ligands (ICI182780, TX, estradiol-17α, E_2–BSA) on TX-stimulated PRL secretion. Moreover, the effect of E_2 on TRH-stimulated PRL secretion in pituitaries collected from estradiol benzoate- and TX-treated OVX rats was studied. It was found that: i) incubation with E_2 supressed the PRL releasing effect of injected TX; ii) whereas coincubation with the pure anti-E type II ICI182780 antagonized the inhibitory effect of E_2, coincubation with the anti-E type I TX did not; iii) estradiol-17α lacked inhibitory action, whereas a dose-dependent inhibitory effect of both E_2 and E_2–BSA was noticed; and iv) TRH stimulatory effect on PRL release in pituitaries from TX-treated rats was blocked by addition of E_2 to the medium. Taken together, these data argue in favor of the presence of specific membrane recognition sites for E in the lactotrope involved in steroid-specific E_2 inhibition of TX-stimulated PRL secretion.

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Introduction

In the rat, secretion of prolactin (PRL) plays a critical role in the maintenance of corpus luteum progesterone secretion during the first half of pregnancy (Morishige & Rothchild 1974, Gibori 1993), mammary tissue through lactation (Tucker 1988), and promoting structural luteolysis in non-functioning corpora lutea (Rothchild 1981, Sánchez-Criado *et al.* 1987). In addition to neurogenic stimuli arising from the environment, estrogen (E) is considered to be the most potent endogenous stimulator of PRL secretion (Neill 1988, Pelletier *et al.* 2003). Classically, E acts by binding to its nuclear receptors in pituitary lactotropes that, in turn, interact with specific DNA sequences to modulate their expression (Watters *et al.* 2000). Ovariectomy and neutralization of circulating E levels with an antiserum reduce PRL secretion (Neill *et al.* 1971, Neill 1972), and administration of anti-E to E-treated ovariectomized (OVX) rats decreases circulating levels of PRL (Gotze *et al.* 1984, Spritzer *et al.* 1996).

Tamoxifen (TX) is a selective estrogen receptor modulator (SERM) that has tissue-selective agonist properties in the rat. TX displays agonist activity at vaginal level, while reducing E action on uterus weight (González *et al.* 2000). At pituitary level, TX displays mixed agonist/antagonist activities. Whereas in the presence of the cognate ligand, TX antagonizes E action on luteinizing hormone (LH) (Tebar *et al.* 1994, Sánchez-Criado *et al.* 2002) and PRL (Lieberman *et al.* 1983, Spritzer *et al.* 1996) secretion, in the absence of the cognate ligand, TX induces GnRH self-priming without affecting basal or GnRH-stimulated LH release in incubated pituitaries (Sánchez-Criado *et al.* 2002), and stimulates PRL secretion (González *et al.* 2000, Bellido *et al.* 2003). Incubated pituitaries from two-week OVX rats injected daily over three days with 3 mg TX exhibit increased PRL release into the medium, as do pituitaries from OVX rats injected with the cognate ligand (Bellido *et al.* 2003). In this model, TX has been found to induce progesterone receptor (PR) expression in gonadotropes (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). Activation of these PR by GnRH-increased intracellular

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cAMP levels, in a P-independent manner (Waring & Turgeon 1992, Turgeon & Waring 1994), elicits GnRH self-priming (Bellido *et al.* 2003). This agonistic effect of TX is silenced by the addition of 10^{-8} M estradiol-17β (E_2) or the membrane-impermeable conjugate E_2–BSA to the medium, suggesting the mediation of a surface E receptor (ER) (Bellido *et al.* 2005). Because GnRH self-priming is a unique response among endocrine cells (Fink 1995), which involves cAMP–PKA signaling pathway cross-talk with the PR (Waring & Turgeon 1992, Turgeon & Waring 1994), it may be that the inhibitory action of E_2 on TX-induced GnRH self-priming is also an unequaled response of gonadotropes. TX stimulates PRL secretion most probably through nuclear ERα (Sánchez-Criado *et al.* 2004, 2005); lactotropes do not express PR (Fox *et al.* 1990, Sánchez-Criado *et al.* 2005) and cAMP is the intracellular mediator for PRL secretion (Neill 1988). For these reasons, the aims of the present study were: first, to determine whether E_2 added to the incubation medium of pituitaries from TX-treated rats suppressed TX-stimulated PRL secretion, and secondly to search for the possible site and mode of action of this paradoxical E_2 effect on lactotropes.

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 05:00 h) at room temperature (22 ± 2 °C) with *ad libitum* access to rat chow and tap water. All rats were OVX under ether anesthesia at random stages of the estrous cycle and included in 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 on laboratory animal care and international law on animal experimentation.

Treatments

In the first experiment, three groups of 12 OVX rats each were daily injected s.c. at 0900 h over three days either with 0.2 ml oil, 25 μg estradiol benzoate (EB; Sigma) or 3 mg of the SERM TX (Sigma). In the second experiment, 48 OVX rats all injected with TX were used. Finally, in the third experiment, three groups of OVX rats treated as in the first experiment were employed. At 0900 h on the first day after treatment, rats from all three experiments were decapitated, the neural lobe discarded and anterior pituitary glands dissected out, divided in halves, and incubated. Doses employed of EB and TX came from previous publications by this laboratory (Bellido *et al.* 2003, 2005, Sánchez-Criado *et al.* 2002, 2004, 2005).

General incubation procedure

Incubation of pituitaries was carried out as previously described (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004). Briefly, halves of anterior pituitaries were incubated for 180 min, after 60 min preincubation, at 37 ° C with constant shaking (60 cycles/min) in an atmosphere of 95% 0_2/5% CO_2. Each incubation tube contained 1 ml of 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. All medium was aspirated every 60 min for quantification of PRL concentrations by specific RIA and replaced with fresh medium containing the corresponding test substance.

Incubation experiments

In the first experiment, 24 hemipituitaries from each of the three groups (oil-, EB- and TX-injected OVX rats) were randomly allocated, in groups of eight, to one of the following three incubation conditions: medium alone, 10^{-8} M 17β estradiol (E_2) (Sigma) and 10^{-7} M TX. The nine groups of hemipituitaries were otherwise submitted to the same incubation protocol. In the second experiment, 96 hemipituitaries from TX-injected rats were randomly allocated ($n = 8$ hemipituitaries/treatment group) to one of the following incubation conditions: medium alone, 10^{-7} M TX; 10^{-7} M ICI182780, a pure anti-E (Tocris Cookson Ltd, Avonmouth, UK); or with: 10^{-10}, 10^{-8}, 10^{-6} M E_2, 10^{-8} M of the stereoisomer 17α E_2 (Sigma); 10^{-10}, 10^{-8}, 10^{-6} M E_2–BSA (Sigma). In addition, pituitaries from TX-treated rats incubated with 10^{-8} M E_2 were coincubated with 10^{-7} M ICI182780 or 10^{-7} M TX. In the final experiment, 16 hemipituitaries from 8 oil-treated rats incubated with medium alone, 16 hemipituitaries from 8 EB-injected rats wereincubated with 10^{-8} M E_2, and 32 hemipituitaries from 16 TX-treated rats incubated either with 10^{-7} M TX (16 hemipituitaries) or 10^{-8} M E_2 (16 hemipituitaries) were co-incubated with 10^{-8} M TRH ($n = 8$ hemipituitaries/treatment group) or without 10^{-8} M TRH ($n = 8$ hemipituitaries/treatment group).

Pituitary weight and protein content determination

Additional OVX rats injected over three days either with 0.2 ml oil, 25 μg EB or 3 mg TX (4 rats/group) were decapitated on the first day after treatments. Their anterior pituitary glands were dissected out, weighed, divided in halves, and processed for protein content following the procedure previously described using bicinchoninic acid (Smith *et al.* 1985).

RIA of PRL

Concentrations of PRL in incubation media were measured in duplicate, by specific RIA, using a double-antibody method with a kit supplied by NIH (Bethesda, MD, USA), and a previously described microassay method (Sánchez-Criado *et al.* 2004). Intra- and inter-assay coefficients of variation were 8% and 9%, respectively, and assay sensitivity was 10 pg/tube. Each PRL value (ng/hemipituitary) was divided by the mean of the corresponding

pituitary protein content and expressed either as ng/mg protein of the reference preparation PRL-rat-RP3, or in arbitrary units, as the area under the curve (AUC) calculated by the trapezoidal rule.

Statistical analysis

PRL data were presented as mean + S.E.M. of eight hemipituitaries/group. Statistical analysis was performed by ANOVA followed by Student–Newman–Keuls multiple range test for comparison among means. Significance was considered at the 0.05 level.

Results

Treatment with EB increased ($P < 0.05$) pituitary weight and pituitary protein content: 15.55 ± 0.93 mg and 0.68 ± 0.04 mg/hemipituitary, respectively, in comparision to oil-injected rats: 10.23 ± 0.47 mg and 0.48 ± 0.02 mg/hemipituitary. Treatment with TX had no effect: 11.00 ± 0.66 mg and 0.49 ± 0.02 mg/hemipituitary.

Regardless of incubation conditions (medium alone, E_2 or TX) pituitaries from OVX rats injected with EB or TX, but not those from OVX rats injected with oil, displayed *in vitro*, increased PRL secretion (Fig. 1). The stimulating effect of E on PRL release was higher ($P < 0.05$) than that of TX (Fig. 1). Overall, incubation conditions did not influence PRL release into the medium, except when E_2 was added to the medium of pituitaries from TX-treated rats. In this case, PRL released into the incubation medium decreased, to such an extent that it did not differ from PRL secretion of pituitaries from oil-injected rats (Fig. 1). The inhibitory effect of E_2 on TX-stimulated PRL secretion was reversed by coincubation with the anti-E ICI182780, but not by TX, while in the absence of E_2 in the incubation medium, ICI182780 or TX alone had no effect on TX-stimulated PRL secretion (Fig. 2). In contrast to the lack of inhibitory effect of the stereoisomer estradiol-17α on PRL release (Fig. 2), both the cognate ligand E_2 and its membrane-impermeable conjugate E_2–BSA inhibited, in a dose-dependent manner, PRL secretion of pituitaries from TX-treated rats (Fig. 2). Moreover, addition of TRH to the medium stimulated PRL secretion in pituitaries from oil-, EB- and TX-treated rats when incubated with the corresponding test substances: medium alone, E_2 or TX, respectively. However, TRH had no stimulatory activity on PRL release in pituitaries from TX-treated rats incubated with E_2 (Fig. 3).

Discussion

Regardless of incubation conditions, data from the present study showed that *in vivo* treatments (oil, EB or TX) determined the *in vitro* pituitary PRL secretory pattern. Thus, addition of E_2 or TX to the medium did not affect PRL release in pituitaries from rats injected either with oil, EB or TX. This was true except when E_2 was added to the incubation medium of pituitaries collected from TX-treated rats, which resulted in complete inhibition of the TX stimulating effect on PRL release. This negative effect of E_2 was strictly dependent on the *in vivo* treatment, since addition of E_2 to the medium of pituitaries from oil- or EB-treated rats had no effect on PRL secretion. The lack of E_2 effect on PRL secretion in pituitaries from oil- or EB-injected OVX rats may be due to E withdrawal in the former (Sánchez-Criado *et al.* 2005) and the occupancy of the complete ER orchestra in the latter.

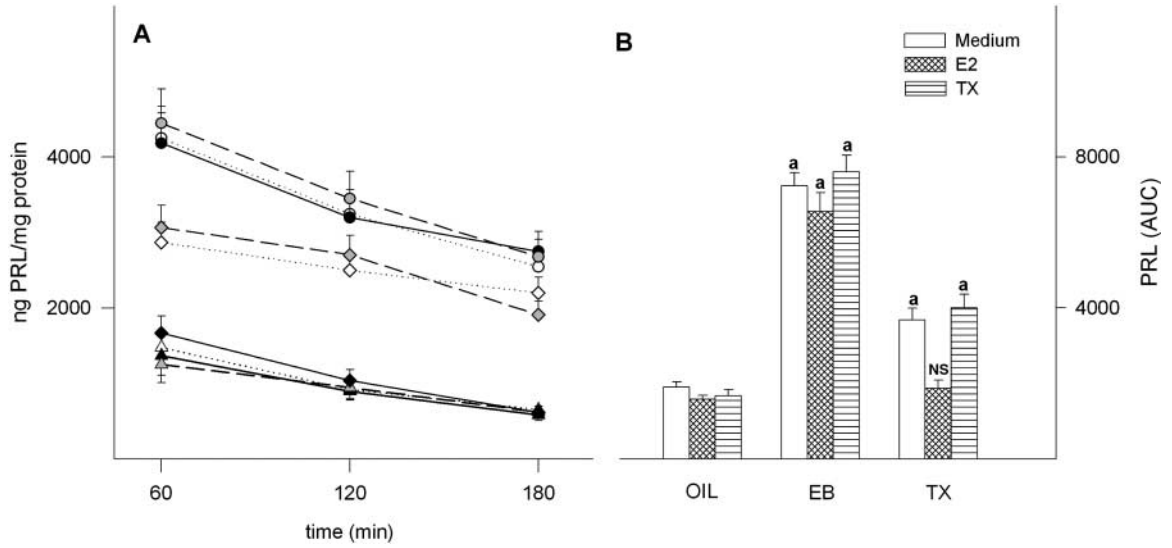

Figure 1 (A) PRL release (ng/mg protein) into incubation medium of pituitaries from two-week OVX rats injected daily over three days with 0.2 ml oil (triangles), 25 μg estradiol benzoate (EB, circles) or 3 mg tamoxifen (TX, diamonds) and incubated for three hours with: medium alone (white), 10^{-8} M estradiol-17β (E_2, black) or 10^{-7} M TX (grey). (B) PRL secretion expressed as area under the curve (AUC) in arbitrary units in the nine experimental groups of the first experiment is represented in B. Values are mean + S.E.M. ($n = 8$ hemipituitaries). a, $P < 0.05$ vs. oil-injected rats; NS, not significant vs. oil-injected rats (ANOVA and Student–Newman–Keuls multiple range test).

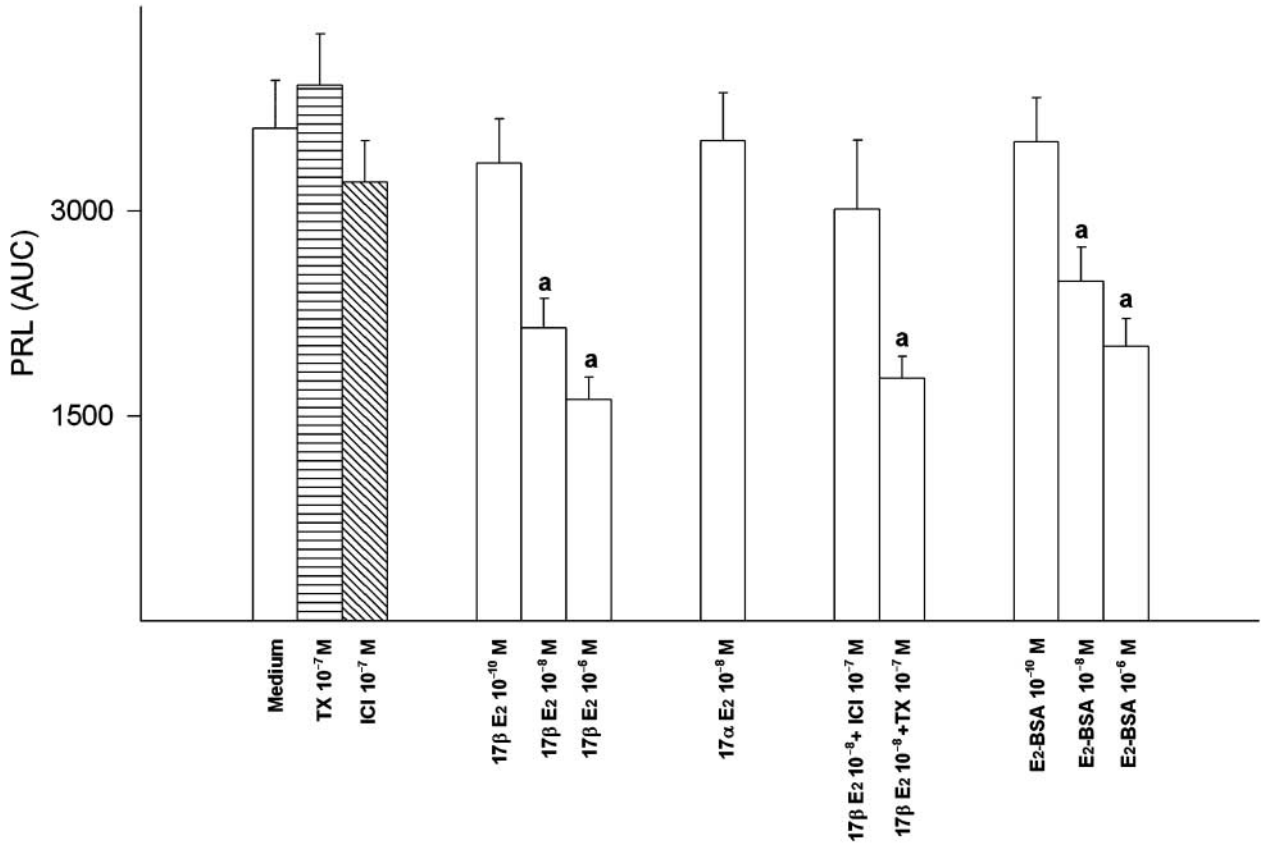

Figure 2 PRL release into incubation medium of pituitaries from two-week OVX rats injected daily over three days with 3 mg TX and incubated for three hours with: medium alone, 10^{-7} M TX; 10^{-7} M ICI182780 (ICI); 10^{-10}, 10^{-8}, 10^{-6} M of E_2; 10^{-8} M 17α-estradiol; 10^{-8} M $E_2 + 10^{-7}$ M ICI; 10^{-8} M $E_2 + 10^{-7}$ M TX; and 10^{-10}, 10^{-8}, 10^{-6} M E_2–BSA. PRL secretion is expressed as area under the curve (AUC) in arbitrary units. 8 hemipituitaries/group. a, $P < 0.05$ vs. controls: medium alone, TX and ICI. (ANOVA and Student–Newman–Keuls multiple range test).

The finding that the agonistic action of the antagonist TX on PRL secretion was antagonized by the cognate agonist is intriguing, as is the fact that physiological concentrations of E_2 in the incubation medium for less than two hours were able to inhibit the stimulatory action on PRL secretion of a three-day pharmacological treatment of TX bound to nuclear ER (Sánchez-Criado *et al.* 2004, 2005). Moreover, the present data indicated that the E_2 blockade of the agonist effect of TX on PRL secretion was reversed by ICI182780, a pure anti-E type II (Smith & O'Malley 2004) that competitively blocks E binding to all known ER (Leondires *et al.* 1999, McEwen & Alves 1999, Navarro *et al.* 2003, Perez-Martin *et al.* 2003), but not by the anti-E type I TX (Smith & O'Malley 2004). Thus, E_2 appeared to inhibit the agonistic effect of TX on lactotropes acting on ER exhibiting both high affinity for the anti-E ICI182780 and extremely low affinity for TX. For these reasons, it seems that this previously undescribed inhibitory action of E_2 on PRL release could be exerted at a non-classical ER in the lactotrope. It has been shown that lactotrope expresses ERα and β isoforms (Mitchner *et al.* 1998, 1999), and probably surface ERα (Christian & Morris 2002, Bulayeva *et al.* 2005). It has also been demonstrated that E_2 binds pituitary membranes with high affinity, and that 4-OH-TX, which is 100-fold more potent than TX as an anti-E (Jordan *et al.* 1988), does not displace E_2 binding to rat pituitary membranes (Bression *et al.* 1986). In contrast, the membrane ER-α-mediated rapid stimulation of PRL release in a pituitary cell line is blocked by ICI182780 (Bulayeva *et al.* 2005). As reported previously for other SERM (Hardy & Valverde 1994, McDonnell 2003), the use of TX in the present experiments may have revealed a novel action of E on rat lactotropes.

In addition to its well defined actions in the nucleus (Watters *et al.* 2000), E has rapid regulatory effects on several membrane associated responses not dependent on changes in gene expression in reproductive tissues (Pietras & Szego 1977). In various cell types, including pituitary cells, there is increasing evidence for non-genomic E effects (Schmidt *et al.* 2000, Kelly & Levin 2001) which, through occupancy of the cell surface ER, triggers membrane associated cytoplasmic signaling cascades that affect cell function (Bression *et al.* 1986). In the present study, incubation of pituitaries from TX-treated rats with increasing concentrations of the analog membrane-impermeable conjugated E_2–BSA, which prevent the steroid from entering the cell (Bression *et al.* 1986), significantly decreased PRL secretion in a dose-dependent

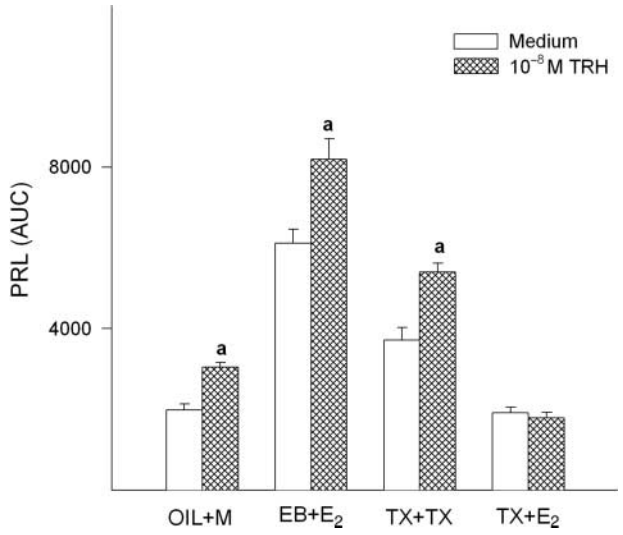

Figure 3 Effect of 10^{-8} M TRH on PRL release into incubation medium of pituitaries from two-week OVX rat injected daily over three days with 0.2 ml oil, 25 μg EB or 3 mg TX and incubated for three hours with: medium alone (M), 10^{-8} M E_2 and 10^{-7} M TX, respectively. Pituitaries from TX-treated OVX rats incubated with E_2 (TX + E_2) were also included. PRL release is expressed as AUC in arbitrary units. 8 hemipituitaries/group. a, $P < 0.05$ vs. non-stimulated controls (ANOVA and Student-Newman-Keuls multiple range test).

manner, as did E_2. It is to be noted that E_2 inhibition of TX-stimulated PRL secretion was evidenced in DMEM containing 0.1% BSA. Since 10^{-8} M of the E_2 stereoisomer estradiol-17α had no inhibitory effect on PRL secretion in TX-treated rats, it seems that the steroid specific E_2 inhibition of TX-stimulated PRL secretion is through specific membrane recognition sites for E in the lactotrope with extremely low affinity for TX.

Pituitary PRL release is strictly dependent on E background (Neill 1988, Mitchner *et al.* 1999). E_2 exerts its excitatory *in vivo* effect on PRL release by enhancing TRH stimulatory effects and suppressing inhibitory influences of dopamine (Neill 1988), respectively. The primary mechanism of E_2, TRH and dopamine actions in the lactotrope involves cAMP as a second messenger (Dannies *et al.* 1976, Barnes *et al.* 1978, Brozmanova *et al.* 1980, Snyder *et al.* 1981, Gautvik *et al.* 1982, Neill 1988). Present data also showed that: (i) addition of TRH to the incubation medium effectively stimulated PRL release in pituitaries regardless of whether it was primed with E or TX, and (ii) that incubation with E_2 annulled the stimulatory effect of the secretagogue TRH in TX-treated rats. There are several lines of evidence suggesting that E_2 inhibition of TX-induced PRL secretion may involve a cAMP/PKA signaling pathway. First, cAMP induces E-like effects in reproductive tissues involving G proteins and second messenger systems in several aspects of E action (Aronica *et al.* 1994, Katzenellenbogen 1996, Yoshioka *et al.* 1999) and TX induces cAMP production in rat pituitary cells (Guelmes *et al.* 2005). Secondly, whereas a stimulatory effect of nanomolar concentrations of E for 60 min was detected in hypothalamic neurons, an inhibitory effect of physiological picomolar E_2 levels exerted on membrane associated receptors in cAMP signaling and GnRH secretion has been demonstrated in hypothalamic neurons (Navarro *et al.* 2003). Such inhibitory responses are abolished by the ER antagonist ICI182780 and mimicked by E_2–BSA (Navarro *et al.* 2003). Thirdly, incubation of pituitaries from TX-treated rats with E_2 inhibits GnRH self-priming but not GnRH-stimulated LH release (Bellido *et al.* 2005). Unlike the GnRH-releasing action of LH, which involves Ca^{2+} and PKC (Stojilkovic *et al.* 1994), GnRH self-priming is a protein-synthesis dependent phenomenon (Fink 1995), that is dependent on cAMP cross-talk with PR in a ligand-independent manner (Waring & Turgeon 1992, Turgeon & Waring 1994).

Taking into account that cAMP is the intracellular mediator for PRL secretion (Neill 1988) and that lactotropes do not express PR (Fox *et al.* 1990, Sánchez-Criado *et al.* 2005), we are now tempted to speculate that the putative ER membrane-mediated inhibitory effect of E_2 on TX-stimulated PRL secretion could be part of a general mechanism of inhibition of cAMP production and/or action. The possible existence in the lactotrope of nuclear and plasma membrane ER, which might prompt cross-talk between intracellular and extracellular effects of E (Blaustein 2004) is suggestive of the existence of a modulatory role in lactotrope function capable of exquisite fine-tuning of E action.

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