Estrogen inhibition of norepinephrine responsiveness is initiated at the plasma membrane of GnRH-producing GT1-7 cells

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Abstract

The modulatory action of estradiol (E₂) on the GnRH network can be exerted indirectly on presynaptic neurons or directly on estrogen receptors (ERs) located within GnRH hypothalamic neurons. Using the GnRH-producing GT1-7 cell line, we have investigated whether E₂ is able to modify the response of these cells to norepinephrine (NE) stimulation. A 48-h exposure of GT1-7 cells to 10 nM E₂ reduced NE-induced cAMP accumulation. However, 15-min exposure was enough to induce this inhibitory action, provided that a hormone-free period of 48 h after steroid treatment was allowed. Furthermore, this effect was mimicked by E₂ coupled to (E-BSA), indicating that it may be exerted through a membrane-mediated mechanism. In addition, competition experiments using E-BSA coupled to fluorescein isothiocyanate (FITC) revealed the presence of cell membrane-binding sites for E₂. Binding of E-BSA

coupled to FITC was blocked by preincubation of cells with either E₂, antiestrogen ICI 162 780, or tamoxifen. Moreover, fluorescence staining of non-permeabilized cells with antibodies against receptors α and β confirmed the presence of both receptor subtypes at the cell membrane. To determine the nature of the ER involved in this response, specific agonists for ER α 4,4',4''-(4-propyl-[¹H]pyrazole-1,3,5-triyl)tris-phenol (PPT) and ER β 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were used. Since PPT, but not DPN, reproduced the effect of E₂, it is suggested that estrogen-induced modulatory action on NE responsiveness was mediated by the ER α isoform. Taken together, these results indicate that E₂ modulates the adrenergic sensitivity of GT1-7 cells by a mechanism compatible with the activation of membrane-associated ERs.

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Introduction

The coordination of episodic release of gonadotrophin-releasing hormone (GnRH) by hypothalamic neurons with GnRH-dependent secretion of pituitary gonadotropins constitutes the central paradigm in the control of mammalian reproduction. The pattern of GnRH secretion is the result of intrinsic oscillatory activity of GnRH neurons and the integration of presynaptic inputs involving a wide variety of neurotransmitters (Kordon *et al.* 1994). In females, ovarian hormones act on the GnRH neuronal network to transynaptically modulate the final output of GnRH into the median eminence (Herbison 1998, Herbison & Pape 2001). Estradiol (E₂), the main endogenous regulator of the hypothalamus–pituitary axis, acts through estrogen receptors (ER) at different levels of the above-mentioned system and induces both excitatory and inhibitory effects on GnRH secretion (Fink 2000). In spite of the sustained idea denying the presence of ERs in GnRH neurons, it is now being accepted that these cells express both membrane-associated and nuclear receptors

and that they are capable of directly responding to changes in circulating E₂ (Malyala *et al.* 2005).

Due to the scattered distribution of GnRH neurons, the characterization of specific estrogen effects and the identification of estrogen-dependent signaling pathways have been extremely difficult. In this respect, immortalized GnRH-producing GT1-7 cells have constituted a valuable tool to study the biology of GnRH neurons (Nunez *et al.* 1998, Vazquez-Martinez *et al.* 2001). By using this experimental model, it has been shown that E₂ is able to directly activate G-protein-dependent signaling cascades, to affect the secretory pattern of GnRH (Navarro *et al.* 2003), and to modulate the response of GnRH-producing cells to classical neurotransmitters (Morales *et al.* 2003, 2005). We previously found that treatment of GT1-7 cells with E₂ for 48 h inhibited the accumulation of cAMP induced by norepinephrine (NE), and that this effect was apparently exerted at a level upstream adenylyl cyclase (AC; Martinez-Morales *et al.* 2001). In the present work, we have investigated whether this modulatory effect can be exerted through estrogen interactions with membrane-binding sites.

Materials and Methods

Cell culture

GT1-7 cells (provided by P Mellon, University of California, San Diego, CA, USA) were grown in a Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS, Sigma–Aldrich), 4.5 g/l glucose, 0.58 g/l glutamine, 3.7 g/l NaHCO₃, 100 µg/ml gentamicin, and 50 µg/ml penicillin. The cells were cultured at 37 °C under 5% CO₂–95% air on 35 mm plates (Falcon, Becton Dickson, NJ, USA). Before any treatment, cells were washed in PBS, pH 7.2, and cultured for 24 h in serum- and phenol red-free Opti-MEM (Gibco BRL).

Reagents

The 17β-E₂, 17α-E₂, tamoxifen (TX), NE, 3-isobutyl-1-methylxanthine (IBMX), BSA, E₂ coupled to bovine serum albumin (E-BSA, 17β-E₂ 6-(O-carboxy-methyl)oxime:BSA; 35 mol E₂/mol BSA), and E₂–BSA–fluorescein isothiocyanate (E-BSA-FITC) were obtained from Biosigma (Madrid, Spain). 4,4',4''-(4-Propyl-[¹H]pyrazole-1,3,5-triyl)tris-phenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were obtained from Tocris (Madrid, Spain). ICI 182 780 was a gift from Astra-Zeneca (Madrid, Spain). The MC-20 polyclonal anti-ERα antibody, which recognizes amino acid residues 580–599 from mouse ERα, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PA1-310B polyclonal anti-ERβ, which recognizes amino acid residues 467–485 from rat ERβ, was obtained from Affinity Bioreagents (Golden, CO, USA). The secondary goat biotinylated anti-rabbit antibody and cyanine-2 dye-conjugated streptavidin were respectively from Vector Laboratories (Burlingame, CA, USA) and Jackson Laboratories (Baltimore, PA, USA). An enzyme immunoassay detection kit for cAMP was obtained from Amersham Biosciences (EIA, RPN225, Little Chalfont, Bucks, UK).

Determination of NE-induced cAMP accumulation

GT1-7 cells were grown in DMEM on 35 mm plates until they reached 60–80% confluence. Then, 24 h before treatments, media were changed to Opti-MEM. In the first set of experiments, cells were exposed to 10 nM E₂ for different times (15 min, 6 h, or 48 h) and, at the end of the hormone incubation period, they were treated with 10 µM NE or the vehicle for 15 min. In the second set of experiments, immediately after incubation with E₂ for the same periods as before, culture medium was replaced by fresh hormone-free medium and incubated for additional periods to reach a total time of 48 h (48, 42, or 0 h), and thereafter the cells were treated with either NE or the vehicle for 15 min. Treatments with E-BSA, PPT, or DPN were performed according to the following experimental protocol: cells were exposed to 10 nM E-BSA, 100 nM PPT, or 100 nM DPN for

15 min, afterwards culture medium was replaced by fresh hormone-free medium and incubated for a total time of 48 h before the addition of NE or the vehicle for 15 min. To eliminate potential contamination of E-BSA with unconjugated E₂, E-BSA solution was prepared by filtration using a Microcon cartridge with a 10 kDa cutoff (meter microcon YM-10, Millipore, Madrid, Spain), following the protocol described by Stevis *et al.* (1999).

In all the cases, the cells were preincubated with the phosphodiesterase inhibitor IBMX (0.25 mM) for 60 min before adrenergic stimulation. After treatments, media were discarded, cells were lysed in 200 µl of 0.25% dodecyltrimethylammonium bromide solution, and frozen at –80 °C until assayed. Cyclic AMP content was determined in duplicate by an enzyme immunoassay kit. The detection limit was 12 fmol, and the inter- and intra-assay variabilities were <15%.

E-BSA-FITC binding assay

Cells cultured on glass coverslips were washed twice with PBS, pH 7.2, and incubated for 1 h at 4 °C in the presence of 10 µM E-BSA-FITC. For competition assays, prior to the addition of E-BSA-FITC, cultures were exposed to E₂, 17α-E₂, TX, or ICI 182 780 (30 and 100 µM each) for 1 h at 37 °C. As a control of unspecific binding, other cells were incubated with 100 µM BSA for 1 h and then exposed to 10 µM E-BSA-FITC. Cells were washed twice with PBS and processed for confocal microscopy visualization. The fluorescent light emitted at the cell membrane was monitored by taking emission images. Relative pixel intensities of individual fluorescent cells were quantified by using Fluoview 1000 software (Olympus FV1000, Barcelona, Spain). Local background was subtracted, and identical experimental and parametrical conditions were used for all treatments.

Membrane-localized ERα and ERβ

Cells cultured on glass coverslips were washed twice with PBS, pH 7.2, and fixed under unpermeabilized conditions in PBS containing 2% PFA, 0.05% glutaraldehyde, and 120 mM sucrose for 30 min at room temperature. The non-permeabilizing fixative conditions employed here have previously been reported to preserve plasma membrane integrity and to prevent anti-ER antibodies from passing across the plasma membrane (Clarke *et al.* 2000). After fixation, cells were washed in PBS and incubated at room temperature with normal goat serum to reduce nonspecific binding. MC-20 and PA1-310B anti-ER antibodies were incubated in PBS for 2 h at room temperature. The secondary biotinylated anti-rabbit antibody (1:200) was incubated for 1 h at room temperature, and staining was revealed by incubation with cyanine-2 dye-conjugated streptavidin (1:500) for 30 min at room temperature. After washing in PBS, cells were mounted in PBS/glycerol (1:1). Negative controls were performed in the absence of primary antibodies. Immunosignals were processed using Fluoview 1000 laser scanning confocal imaging system.

Statistical analyses

Data are expressed as mean $\pm$ S.E.M. Significant differences among groups were tested by one-way ANOVA followed by the Tukey's *post hoc* test. Statistical significance is indicated in the figures from $P < 0.05$.

Results

Modulation of NE-induced cAMP accumulation by E₂

As previously reported (Martinez-Morales *et al.* 2001), pretreatment of GT1-7 cells with E₂ (10 nM) for 48 h, but for neither 15 min nor 6 h, reduced NE-induced cAMP accumulation when the neurotransmitter was applied immediately after hormone exposure (Fig. 1A). In the second experiment, cells were pretreated with the same

concentration of E₂ for 15 min, 6 h, or 48 h but, in this case, a total period of 48 h was allowed prior to NE stimulation. Thus, when exposure to E₂ was shorter than 48 h (15 min or 6 h), culture medium was replaced by fresh hormone-free medium. Interestingly, short exposures to the same concentration of E₂ produced an equivalent inhibition of NE-induced cAMP accumulation when the effect of E₂ had a total latency of 48 h (Fig. 1B).

Estrogen modulatory effect is initiated at the plasma membrane

Even though estrogen effect on NE-induced cAMP accumulation has a long latency, which is compatible with a genomic mechanism, this action could be initiated at the plasma membrane level, as a short exposure to E₂ is enough to inhibit adrenergic stimulation. To test this hypothesis, we used 17 β -E₂ coupled to BSA (E-BSA), a membrane-impermeant analog of E₂. Cells were pretreated with E-BSA and E₂ for 15 min and then, after 48 h incubation in hormone-free medium, stimulated with NE. As shown in Fig. 2, a similar inhibitory effect of both compounds on NE-induced cAMP accumulation was observed. BSA alone did not show any effect on cAMP levels induced by NE (data not shown), thus demonstrating the specificity of E-BSA action.

Impermeant conjugate E-BSA-FITC binds to specific sites of the plasma membrane

The fact that the membrane-impermeant molecule E-BSA reproduces the effect of E₂ suggests the presence of estrogen plasma membrane-binding sites in GT1-7 cells. To analyze the existence and specificity of these binding sites, we exposed the cells to E-BSA coupled to FITC. After incubation with

Figure 1 Effect of 17 β -estradiol on NE-induced accumulation of cAMP. (A) Cells were pretreated with 10 nM E₂ or the vehicle (0.001% ethanol) for 15 min, 6 h, or 48 h, and then the cells were treated with norepinephrine (NE, 10 μ M) or vehicle (Ctrl) for 15 min. (B) Cells were pretreated with 10 nM E₂ or the vehicle (0.001% ethanol) for 15 min, 6 h, or 48 h, and the cells were stimulated with NE after a total period of 48 h for each group. Cyclic AMP was determined in duplicate by an enzyme immunoassay kit, and each bar represents the mean $\pm$ S.E.M. of six plates per group. ^a $P < 0.001$ versus Ctrl; ^b $P < 0.01$ versus NE in the vehicle group.

Figure 2 Effect of 17 β -estradiol and E-BSA on NE-induced accumulation of cAMP. Cells were pretreated with 10 nM E₂, 10 nM E-BSA, or the vehicle (0.001% ethanol) for 15 min, and after 48 h, they were stimulated with 10 μ M NE or the vehicle (Ctrl) for 15 min. Cyclic AMP was determined in duplicate by an enzyme immunoassay kit, and each bar represents the mean $\pm$ S.E.M. of six plates per group. ^a $P < 0.001$ versus Ctrl; ^b $P < 0.01$ versus NE in the vehicle group.

this compound, $\approx 90\%$ of the cells exhibited fluorescent labeling at the cell membrane, which was unaffected by preincubation with an excess (100 μM) of BSA (Fig. 3A and B). The binding of E-BSA-FITC was partially blocked by a threefold excess of either E_2 (Fig. 3C), antiestrogen ICI 182 780 (Fig. 3E), or selective ER modulator TX (Fig. 3G). A nearly complete blockade of immunostaining at the outer cell membrane was obtained by preincubation of cells with 100 μM E_2 , ICI 182 780, or TX (Fig. 3D, F, and H respectively). However, 100 μM of the relatively inactive stereoisomer $17\alpha\text{-E}_2$ did not modify the binding of E-BSA-FITC to the plasma membrane (Fig. 3J), thereby suggesting that these binding sites present in GT1-7 cells are specific for E_2 . Quantification of the percentage of labeled spots revealed a dose-dependent reduction in the binding of E-BSA-FITC at the cell membrane with increasing concentrations of either E_2 , ICI 182 780, or TX (Fig. 3I).

Immunoreactivity for ER α and ER β at the plasma membrane domain

Since estrogen seems to bind to specific receptors at the plasma membrane of GT1-7 cells, we further explored the putative structural homologies of ER α and ER β with these membrane-related E_2 -binding molecules. Non-permeabilized GT1-7 cells were incubated with either MC-20 or PA1-310B antibody directed to the carboxy-terminal region of ER α and ER β respectively. The visualization of cells by confocal microscopy revealed fluorescence staining for both ER α (Fig. 4A and B) and ER β (Fig. 4C and D). These results suggest that binding sites for E_2 at the plasma membrane of GT1-7 cells share structural homologies with classical ERs, ER α and ER β , at least at their carboxy-terminal domain.

Specific activation of ER α mimicked the effect of E_2

Since the plasma membrane of GT1-7 cells exhibits binding sites for E_2 sharing structural homologies with both classical ERs, we sought to determine the potential role of ER subtypes in mediating the modulation of E_2 on NE-induced cAMP accumulation by using the selective ER α and ER β agonists, PPT and DPN respectively. Cells were pretreated with E_2 , PPT, or DPN for 15 min and then, after 48 h incubation in hormone-free medium, stimulated with NE. As shown in Fig. 5, 100 nM PPT, but not DPN, produced a complete inhibition of cAMP accumulation induced by adrenergic stimulation. This total blockade can be explained by the high concentration of PPT that we have used in our assay (100 nM), because this specific agonist has a median effective concentration EC_{50} of ca. 1 nM on ER α (Stauffer *et al.* 2000). These results indicate that the effect of E_2 is mediated by the activation of the ER α subtype.

Discussion

We have previously reported that the accumulation of cAMP induced by NE in GT1-7 cells is reduced by 48 h pre-exposure to nanomolar concentrations of E_2 (Martinez-Morales *et al.* 2001). Since E_2 did not affect the accumulation of cAMP induced by forskolin, a direct activator of AC, it was suggested that the final target of estrogen action was located upstream the enzyme responsible for second messenger production. Moreover, the temporal pattern of hormone treatment was compatible with a genomic mechanism and a classical ER was thought to be involved. However, in the present study, we show that short exposures to E_2 (15 min) are sufficient to reduce NE-induced cAMP accumulation in GT1-7 cells, provided that a 48-h period is allowed prior to adrenergic stimulation. Furthermore, estrogen exerts its modulatory effect through a mechanism that is initiated at the plasma membrane, since the effect of E_2 is reproduced by equivalent doses of E-BSA, a compound that is unable to cross the plasma membrane (Caldwell & Moe 1999, Stevis *et al.* 1999). We have also found that GT1-7 cells exhibit membrane-binding sites that are specific for E_2 , which are blocked by related compounds in a concentration-dependent manner. In addition, in non-permeabilized cells, confocal microscopy revealed fluorescence staining for both ER α and ER β when using antibodies against their carboxy-terminal domain. Even though both isoforms of ER are detected at the plasma membrane, our results indicate that E_2 modulates the response of GT1-7 cells to adrenergic stimulation through selective activation of ER α , since PPT, but not DPN, reproduced the effect of E_2 on NE-induced cAMP accumulation. Taken together, these results indicate that, at nanomolar concentrations, E_2 is able to modulate non-adrenergic responsiveness in GnRH-producing GT1-7 cells by a mechanism compatible with hormone interactions with membrane-associated ER α .

Rapid effects of E_2 noticeable within seconds or minutes have been extensively reported (Nadal *et al.* 2001). Depending on the cell type, estrogen exposure elicits rapid activation of different signaling pathways and second messenger-dependent kinases through the binding of estrogen to the plasma membrane (Pedram *et al.* 2002, Zhang *et al.* 2002, Guerra *et al.* 2004). Moreover, changes in transcription from ERE-containing genes or genes lacking ERE promoters can be led by membrane-initiated hormone actions (reviewed in Vasudevan & Pfaff 2007). Since estrogen-responding cells may contain both membrane-associated and classical nuclear ERs, it is conceivable that different signaling cascades may be integrated to promote the final cell response (Levin 2005, Marin *et al.* 2005).

With respect to the nature of membrane-associated receptors, several studies have reported that they may be the same molecules rather than their intracellular counterparts, derived from the same transcripts other than ER α and ER β (Razandi *et al.* 1999), and responsible for rapid signaling in

Figure 3 E-BSA-FITC binding of GT1-7 cells and competition with E₂, ICI 182 780, tamoxifen (TX), and 17 α -estradiol. Specific fluorescence staining was detected at the membrane of cells with 10 μ M E-BSA-FITC (A). This labeling remained unaffected with a tenfold excess of BSA (B) and partially competed off with 30 μ M E₂ (C), ICI 182 780 (E), or TX (G). Doses of 100 μ M of the same compounds completely blocked the staining of E-BSA-FITC (D, F, and H respectively), whereas 100 μ M 17 α -estradiol did not affect the labeling (J). (I) Quantification of labeled pixels in cells exposed to E-BSA-FITC and E₂, ICI 182 780, or TX for the different doses used. Values are referred to the percentage of pixel intensities in E-BSA-FITC cells used as a control. ^a*P* < 0.05 and ^b*P* < 0.001 versus E-BSA-FITC. Number of analyzed cells per group is 25. Scale bar = 10 μ m.

Figure 4 Membrane localization of ER α and ER β in GT1-7 cells. Cells were fixed under non-permeabilizing conditions and incubated with the anti-ER α antibody MC-20 (A and B) and the anti-ER β PA1-310B (C and D). Scale bar = 10 μ m.

different cell types (Razandi *et al.* 2003, 2004, Marin *et al.* 2006). On the contrary, other investigations have revealed membrane-mediated actions of E₂ exerted through the activation of proteins other than classical ER. In line with this possibility, some fragmentary evidence points to the existence of a G-protein-coupled receptor (GPR30), which is able to bind estrogen and activate intracellular signaling pathways (Filardo *et al.* 2002, Revankar *et al.* 2005, Thomas *et al.* 2005). Thus, it remains to be determined whether the vast majority of rapid estrogen effects are only dependent on classical ERs associated with the plasma membrane or, alternatively, that other estrogen-binding proteins may contribute to overall cell responses (Pedram *et al.* 2006).

The presence of ERs in hypothalamic GnRH neurons has been a controversial issue for a long time (Herbison 1998, Herbison & Pape 2001). However, this view changed

completely when a second subtype of ER, namely ER β , was found (Kuiper *et al.* 1996). It has been shown that certain populations of murine GnRH neurons express both the mRNA encoding ER β (Skynner *et al.* 1999, Hrabovszky *et al.* 2000, Sharifi *et al.* 2002) and the protein (Hrabovszky *et al.* 2001, Kallo *et al.* 2001, Legan & Tsai 2003). On the other hand, immortalized GnRH-producing GT1-7 cells express both ER α and ER β transcripts and proteins (Butler *et al.* 1999, Roy *et al.* 1999, Martinez-Morales *et al.* 2001), and exhibit plasma membrane estrogen-binding sites (Morales *et al.* 2003). In the present study, the fact that E-BSA mimics the effect of E₂ on NE-induced cAMP accumulation suggests the involvement of membrane-associated ERs. In addition, using E-BSA conjugated to FITC, we have shown the existence of specific estrogen-binding sites at the plasma membrane which are competed, in a dose-dependent

Figure 5 Effect of 17 β -estradiol, PPT, and DPN on NE-induced accumulation of cAMP. Cells were pretreated with 10 nM E₂, 100 nM PPT, 100 nM DPN, or the vehicle (0.001% ethanol) for 15 min, and after 48 h, they were exposed to 10 μ M NE or the vehicle (Ctrl) for 15 min. Cyclic AMP was determined in duplicate by an enzyme immunoassay kit, and each bar represents the mean $\pm$ S.E.M. of six plates per group. ^a $P < 0.001$ versus Ctrl; ^b $P < 0.05$ versus Ctrl; ^c $P < 0.01$ versus NE in the vehicle group.

manner, by either E₂, ICI 182 780, or TX. Although E₂ coupled to BSA has been widely used to mimic a membrane-limited action for E₂, there has been some controversy over the possible release of free E₂ from the conjugate and the possibility of endocytosis of the conjugate itself. In our study, before the application to the cells, E-BSA solution was filtered to remove potential free E₂. Furthermore, we can rule out the endocytosis of E-BSA since the incubation of GT1-7 cells with E-BSA-FITC for 1 h, at either 37 or 4 °C, did not provoke the entrance of the conjugate into the cells (Morales *et al.* 2003).

Fluorescence staining of non-permeabilized GT1-7 cells with antibodies against the ligand-binding domain of ER α and ER β has confirmed the presence of these isoforms as previously described by other groups (Navarro *et al.* 2003). The modulatory action of PPT, a highly selective and specific ER α agonist, on the NE-induced cAMP accumulation led us to conclude that the effect of E₂ on adrenergic responsiveness in GT1-7 cells is exerted through the interaction with membrane-associated ER α .

A number of ER α isoforms have been involved in rapid and alternative estrogen signaling. Flouriot and colleagues have identified a 46 kDa isoform that lacks exon 1 and, consequently, the N-terminal AF-1 region (Flouriot *et al.* 2000). This isoform has been identified at the plasma membrane of several cell types, where it modulates membrane-initiated estrogen actions (Li *et al.* 2003). Recently, a new spliced variant of ER α , hER- α 36, has been identified (Wang *et al.* 2006). This protein lacks the two transcriptional activation domains, and it is predominantly associated with the plasma membrane.

In mammals, the pattern of GnRH secretion is under the control of estrogen acting at different levels of the hypothalamic neuronal network (Herbison 1998). Studies from immortalized GT1-7 cells have helped clarify some of the mechanisms that may participate in direct estrogen modulation of GnRH neuronal activity. Estrogen has been shown to exert both inhibitory and stimulatory, dose-dependent effects on GnRH pulsatile secretion and cAMP formation (Navarro *et al.* 2003). In addition, E₂ also modulates acetylcholine-induced calcium signals by a membrane-mediated mechanism involving cGMP formation and PKG-dependent inositol triphosphate receptor phosphorylation (Morales *et al.* 2003, 2005). We now report for the first time that estrogen modulation of noradrenergic responsiveness is initiated at the plasma membrane of GT1-7 cells. Since GnRH neurons are the target of multiple presynaptic inputs, estrogen-dependent modulation of neurotransmitter sensitivity can be an efficient way to adapt the response of this neuronal system to gonadal steroids during mammalian ovarian cycles.

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