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17β-Estradiol stimulates the translocation of endogenous estrogen receptor α at the plasma membrane of normal anterior pituitary cells

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ABSTRACT

In the present work we aimed at identifying ERα in the plasma membrane of normal anterior pituitary cells and investigated if 17β-estradiol was able to induce their subcellular redistribution. Our results show that about 8% of anterior pituitary cells expressed ERα in the plasma membrane, with the geometrical mean fluorescence intensity being increased after steroid hormone treatment. 17β-Estradiol and the selective ERα agonist PPT induced an increase of ERα expression in the plasma membrane and activated the PKCα/ERK 1/2 pathway in a time-course not compatible with genomic actions, thus supporting the notion of membrane-initiated effects. These findings suggest that 17β-estradiol stimulates the translocation of endogenous ERα to the plasma membrane, consequently modulating this ER pool and leading to cellular biological effects in normal anterior pituitary gland.

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1. Introduction

In order to mediate their biological effects, estrogens interact with classical intracellular estrogen receptors (ER) α (Green et al., 1986) and β (Gustafsson, 1999), which function as ligand-dependent transcription factors. Although it is known that this hormone activates membrane-associated ER (Pietras and Szego, 1977; Bulayeva et al., 2004; Marin et al., 2009) there are several intriguing questions not elucidated about this topic, being a very active area of original researches and reviews (Warner and Gustafsson, 2006; Levin, 2009b, 2011).

Several investigations have identified the classical intracellular ER at the cell-surface of different cells: GH3B6F10 pituitary tumor cells (Norfleet et al., 1999), lactotrophs (Gutiérrez et al., 2008), hypothalamic neurons (Gorosito et al., 2008), hypothalamic astrocytes (Bondar et al., 2009), and blood lymphocytes (Pierdominici et al., 2010). Moreover, there is abundant evidence about the rapid effects exerted by estradiol, which are incompatible with classical genomic effects, suggesting the presence of estradiol binding sites in the plasma membrane. Related to this, it has been shown that estradiol induces rapid activation of phosphatidylinositol-3 kinase, mitogen-activated protein kinase (Carrer et al., 2005; Dominguez et al., 2007), protein kinase A, protein kinase C (PKC) (Dewing et al., 2008), and the nitric oxide/guanylyl cyclase pathway (Gutiérrez et al., 2010), as well as leading to increased calcium levels (Bulayeva et al., 2005) and cAMP accumulation (Aronica et al., 1994) in different tissues.

ER mobilization within the cell is a widely debated issue. Classically, the ER was shown to be constantly shuttling between the nucleus and cytoplasm, although under steady-state conditions it was detected predominantly in the cell nucleus in both the absence and presence of estradiol (Dauvois et al., 1993). However, following the discovery of a new pool of ER located in the plasma membrane, their mobilization in and out of the plasma membrane has become a topic of active research investigation. Some studies have demonstrated ERα translocation between membrane and nuclear compartments (Lu et al., 2002) and ERα and β mobilization to the plasma membrane being shown in Sertoli cells (Lucas et al.,

Abbreviations: ER, estrogen receptor; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol, selective ERα agonist; ICI 182780, (7a,17b)-7-[9-[(4,4,5,5,5pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; PKC, protein kinase C.

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2008). Also, in hypothalamic astrocytes, traffic in and out of the plasma membrane regulated by estradiol has been reported (Bondar et al., 2009).

In the pituitary gland, both the ERα and β subtypes have been identified in the majority of the hormone-producing cells (Keefer et al., 1976; Mitchner et al., 1998), which mediate a diversity of physiological responses, and are relevant for the control of cell growth and function. It has also been demonstrated that estradiol stimulates prolactin secretion (Bulayeva et al., 2005) and modulates the lactotroph cell proliferation, thereby inducing mitogenic/antimitogenic actions (Gutiérrez et al., 2005, 2008; Kawashima et al., 2002) and apoptosis of anterior pituitary cells (Zaldivar et al., 2009; Zárate and Seilicovich, 2010). Although some evidence suggests that ERα is the main mediator of the estradiol effects in the pituitary gland, little is known about the presence of ER in the plasma membrane and its subcellular mobilization in pituitary cells. Taking account our previous report that showed the ERα in the plasma membrane of lactotroph, in the present work we sought strengthen this finding, establishing the effects of 17β-estradiol on the subcellular distribution of ERα and their functionality in normal anterior pituitary cells.

2. Materials and methods

2.1. Animals

Three-month-old female rats of the Wistar strain were used. Large pools of anterior pituitary glands from around 40 virgin female rats taken at random cycle stages were utilized for each primary anterior pituitary cell culture. All rats were bred and housed at the Animal Research Facility of the National University of Córdoba, in air-conditioned quarters, under a controlled photoperiod (14L:10D) with free access to commercial rodent food and tap water. Animals were kept in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (1996) and the local Institutional Animal Care Committee.

2.2. Dissociation of anterior pituitary cells

The rats were decapitated within 10 s of removal from their cage, thus avoiding any stress or external stimuli. Anterior pituitary glands were rapidly removed and processed for cell cultures. The protocol for the dissociation of pituitary cells has been described previously (De Paul et al., 1997). Briefly, anterior pituitaries excised from female rats were placed in minimal essential medium for suspension culture (SMEM), before being minced, digested with 0.4% trypsin and dispersed with Pasteur pipettes. The cell yield was $1.5–2 \times 10^6$ per pituitary, and the cell viability, tested with Trypan Blue exclusion, was always better than 90%. The final suspension was adjusted to 1×10^6 cells/ml of medium. For immunofluorescence by confocal microscopy, the cells were seeded on glass coverslips (13 mm) at a density of 2×10^5 cells/well and placed at the bottom of 24-well culture plates (Corning, NY). For the other techniques, the cells were plated on 6-well culture plates (Corning, NY) at a density of 5×10^5 cells/well. Then, the cells were maintained in DMEM supplemented with 4% fetal calf serum and 8% horse serum (Gibco, NY) in an oven with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3 days.

All culture media were filtered through 0.2 μm Nalgene membranes (Nalge Company, NY), with the cell culture grade reagents being obtained from the Sigma Chemical Co., St. Louis, MO.

2.3. Cell treatments

After 3 days of culture, the medium was discarded and replaced with DMEM without Phenol Red or serum, supplemented with hydrocortisone (100 μg/L), 3,3′-triiodothyronine (400 ng/L), transferrin (10 mg/L) and sodium selenite (5 μg/L) for 24 h. Then, the cells were stimulated with 17β-estradiol (10 nM, Sigma, St. Louis, MO), the selective ERα agonist, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol (PPT; 10 nM; Tocris Cookson Inc., Ellisville, MO) or estradiol-BSA (10 nM; β-estradiol 6-*O*-carboxymethyloxime: BSA, Sigma, St. Louis, MO). A stock solution of estradiol-BSA was made by dissolving the powder in buffer (50 mM Tris–HCl, pH 8.5) at 1 mg/ml. An aliquot of the solution was added to a centrifugal filter unit with a MW cut-off of 3000 (Millipore) and subjected to centrifugation at 13000*g* for 30 min. The filtrate was recovered and the retentate was washed 3 times with 400 μl of buffer Tris–HCl.

The anterior pituitary cell cultures were pre-treated for 30 min with ICI 182780 (100 nM, Sigma, St. Louis, MO) and then E2, PPT, or E2-BSA were added and incubated for another 15 min. Concentrations of this inhibitor were chosen from preliminary experiments, following a previous report (Gutiérrez et al., 2010).

The inhibitor of calcium-dependent PKC Gö6976, (used to inhibit the PKCα isozyme (1, 5 and 10 μM; Calbiochem, San Diego) and the inhibitor of mitogen-activated kinase effector kinase (MEK) PD98059 (1, 50 and 100 μM, Calbiochem, San Diego) (used to inhibit ERK1/2 activation) were added for 30 min, and then the cultures were stimulated with 17β-estradiol for 15 min (Petiti et al., 2010).

2.4. Antibodies

Anti-ERα: MC-20, directed to the C-terminal of the ERα (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); anti-total ERK1/2 (1:200, Santa Cruz, Biotechnology, Santa Cruz, CA) and phosphorylated (1:700, Sigma, St. Louis, MO); anti-PKCα (1:300, Santa Cruz, Biotechnology, Santa Cruz, CA); anti-prolactin (1:3,000, Dr. A. Parlow, National Hormone and Pituitary Program, Torrance, CA); anti-NF-κB (1:1500, Abcam, Cambridge, MA); anti-β-amyloid precursor-like protein 2 (APLP2, 1:500, Calbiochem, San Diego); anti-β actin (1: 5000, mouse monoclonal antibody; Sigma–Aldrich, St. Louis, MO), anti-insulin receptor (1:300, Santa Cruz Biotechnology).

2.5. Immunoelectron microscopy (pre-embedding method)

In order to identify the presence of membrane ERα in normal anterior pituitary cells, a procedure for immunolabeling in intact cells was performed (Gutiérrez et al., 2007). This technique, designed for antigen labeling on the cell surfaces prior to fixation, allows excellent preservation of the antigen and cellular fine-structure, thereby improving the immunocytochemistry and the ultrastructural morphology. Anterior pituitary cells were rinsed with Hanks solution, and the unspecific endogenous antigens were blocked with 1% PBS–BSA for 15 min at 37 °C. The cells were then incubated with the primary antibody for 1 h at 37 °C, rinsed with Hanks solution, treated with 1% PBS–BSA for 15 min at 37 °C and incubated with anti-rabbit secondary antibody conjugated to 15 nm colloidal gold particles (1:20 dilution, Electron Microscopy, Hatfield, PA) for 1 h at room temperature. After the immunocytochemical reaction, the cells were scraped from the wells, centrifuged, and fixed in Karnovsky fluid (1.5% glutaraldheyde, 1.5% formaldehyde in 0.1 M cacodylate buffer). Then, fixed cells were centrifuged and the pellets treated with 1% OsO_4 for 1 h, and then stained *in bloc* with 1% uranyl acetate in 0.1 M acetate buffer, pH 5.2, for 20 min. After dehydration in a series of graded cold acetones, the cells were embedded in Araldite. Thin sections, cut using a JEOL ultramicrotome with a diamond knife, were stained with uranyl acetate/lead citrate and examined in a Zeiss LEO 906-E electron microscope.

To validate the specificity of the ERα immunostaining, both negative and absorption (blocking peptide) controls were performed by applying the same protocol but replacing primary antibody with 1% PBS–BSA or with antibody pre-absorbed with purified antigens. A blocking peptide was used by the pre-incubation of antibody dilutions, with a threefold excess of the respective antigen peptide (ERα, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C.

For the morphometric analysis of the ERα expression at the cell surface by electron microscopy, 500 micrographs of lactotroph cells from each experimental condition were taken. The number of gold particles attached on the plasma membrane was counted using Image J. and a statistical analysis was performed.

2.6. Confocal laser scanning microscopy

Anterior pituitary cells from different experimental groups were fixed in 4% formaldehyde, permeabilized in 0.25% Triton X-100 in PBS, blocked for 1 h in 3% PBS–BSA and incubated with the primary antibodies (anti-ERα or anti-PKCα) for 1 h. Then, these cells were washed, and further incubated with an Alexa 488 anti-rabbit or Alexa 594 anti-mouse secondary antibody (1:1000; Invitrogen; Carlsbad, USA) for 1 h.

In non-permeabilized anterior pituitary cells, the plasma membranes were stained with Concanavalin A Alexa Fluor 488 conjugate (100 μg/ml, Invitrogen, Eugene, OR), which selectively binds to α-mannopyranosyl and α-glucopyronosyl residues, common to many membrane glycoproteins.

To validate the specificity of the ERα immunostaining, negative and absorption (blocking peptide) controls were performed, as was described above.

Images were then obtained using an inverted confocal laser scanning microscope FluoView FV 1000 (Olympus; Tokyo, Japan). An Ar-ion 488 nm laser was used for excitation of green fluorescence, and a 543 nm one for excitation of red fluorescence. Serial *z*-axis sections were collected with a 60 or 100× objective. Analysis of confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer.

2.7. Analysis of cell-surface proteins by biotinylation

The cell-surface proteins were labeled according to Gorosito et al. (2008) with modifications. Briefly, anterior pituitary cell cultures were washed three times with ice-cold PBS buffer, pH 8, and then incubated with the membrane impermeable sulfo-NHS-biotin (Pierce, Rockford, IL) at a final concentration of 0.3 mg/ml in PBS, pH 8, at 4 °C for 20 min. After biotinylation, the cultures were washed three times with ice-cold PBS, and harvested with lysis buffer containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (all inhibitors were from Sigma, St. Louis, MO). The cellular extracts were sonicated and incubated with avidin-agarose beads (Pierce, Rockford, IL) for 2 h at 4 °C. Then, the extracts were centrifuged at 500*g* for 10 min at 4 °C, the supernatant (200 μl) was separated and the pellet was washed three times with 1 ml of lysis buffer. All the pellets from each experimental condition were concentrated (30 μl) and run in one lane. The proteins from the supernatant fractions were quantified and 30 μg per lane were run in 12% acrylamide gel. Both pellet and supernatant fractions were analyzed by Western blot using specific antibodies.

2.8. Preparation of cellular homogenates for immunoblot analysis

For the total extracts, anterior pituitary cells rinsed with PBS were lysed on ice by the addition of 200 μl of lysis buffer followed by the scraping and transfer of the lysate to a centrifuge tube placed on ice. After 30 min, the lysates were centrifuged at 14,000*g* for 10 min at 4 °C to pellet the Igepal CA-630-insoluble material, and the supernatants were withdrawn and stored in aliquots frozen at −80 °C until required.

2.9. Protein measurement

Protein content was measured using a BioRad kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA).

2.10. Western blot analysis

The samples were run in 12% acrylamide gel (Sigma, St. Louis, MO). To estimate the corresponding molecular weights, Full Range Rainbow Molecular Weight Marker was run in parallel (Amersham-Life Science, Bucks, England). The proteins were transferred to a nitrocellulose membrane, and non-specific binding was blocked with PBS containing 5% nonfat dried milk and 0.1% Tween 20 (blocking buffer) at room temperature. The membranes were rinsed and incubated for 2 h with the primary antibodies (anti-ERα, t-ERK 1/2, p-ERK 1/2, NF-κB and APLP2). These blots were thoroughly rinsed in PBS/0.1% Tween-20, and the HRP-coupled secondary antibody (1: 5000, Jackson Immunoresearch Labs Inc., West Grove, PA) was revealed with ECL Western blot detection reagents (Amersham Biosciences, Bucks, UK), following the manufacturer's instructions. Emitted light was captured on Hyperfilm (Amersham-Pharmacia-Biotech, Bucks, UK). Signals were scanned and quantified with Scion Image software (V. beta 4.0.2, Scion Image Corp., Frederick, MD) at three different exposure times. The expression of β-actin and insulin receptor was used as an internal control to confirm the equivalent total protein loading.

2.11. Flow cytometry

Living dispersed anterior pituitary cells were incubated with anti-ERα or an appropriate isotype control (BD Biosciences) for 30 min at 4 °C and then incubated with secondary antibody Alexa Fluor 647 (1:1000, Invitrogen, Eugene, Oregon) for 30 min at 4 °C. After this time, the cells were fixed (CITOFIX, BD Biosciences Pharmiogen, San Diego, CA) for 20 min at 4 °C, before being washed, resuspended in filtered PBS and analyzed by flow cytometry (1×10^5 events/experimental treatment, Ortho Diagnostic System, Raritan, NJ). The data analysis was performed using the FlowJo software (Tree Star, Inc., Ashland, OR).

To quantify the ERα expression on the cell surface of lactotroph cells, other cell batches were labeled for ERα as described above, and then permeabilized with Perm/Wash (BD Biosciences Pharmiogen, San Diego, CA) and incubated with primary antibody (anti-PRL) for 30 min at 4 °C. Then, the cells were incubated with secondary antibody FITC (1:300, Santa Cruz Biotechnology) for 30 min at 4 °C and finally processed and analyzed by flow cytometry as described above.

The percentage of membrane ERα (mERα) positive cells was calculated using the equation [(mERα+/total cells] × 100 or [(mERα + PRL+)/total PRL + cells] × 100, and the variations in the intensity levels of mERα were determined using the geometric mean fluorescence intensity (Gmean).

2.12. Data analysis

The statistical analysis was carried out on three replicates measured on three independent cell cultures using ANOVA. This was followed up by using the Fisher test of the InfoStat software package. The significance level was chosen as $P < 0.05$.

3. Results

3.1. ERα is localized in the plasma membrane of anterior pituitary cells

From immuno-electron-microscopy of intact cells, ERα was found to be present on the cell surface of lactotroph cells, showing colloidal gold particles attached to the plasmalemma. In basal conditions the morphometric analysis showed 6.07 ± 1.67 colloidal gold particles in 10 μm of plasma membrane, with a significant increase occurring after 15 min of 17β-estradiol treatment and reaching values of 11.69 ± 1.46 (Fig. 1A–D). Immunocytochemical controls validated the specificity of the primary antiserum, with no immunolabeling being found after the omission of the primary antibody or after its pre-absorption with purified antigen (Fig. 1E).

To investigate further the association of ERα with the plasma membrane and to determine whether its localization was regulated by 17β-estradiol, anterior pituitary cells were submitted to cell surface biotinylation. As shown in Fig. 2A, Western blot analysis revealed the presence of ERα in both the pellet fraction (containing the cell surface biotinylated proteins), and the supernatant fraction (with the intracellular unbiotinylated proteins). In the pellet, only one ERα-immunoreactive band at 66 kDa was identified, corresponding to the ERα full-length. At basal conditions, a weak expression of ERα was shown, which was increased by 17β-estradiol or PPT after 15 min stimulation. In the supernatant, two ERα-immunoreactive bands were observed, at ≈66 kDa and ≈55 kDa, with the band of the lower position possibly corresponding to the splicing variant of ERα (Hasbi et al., 2005; Gorosito et al., 2008).

To demonstrate that equivalent total protein loading took place in the pellet and supernatant fractions, insulin receptor and β-actin protein expression were used respectively, considering that in a previous report we described that 17β-estradiol was unable to increase the insulin receptor expression (Gutiérrez et al., 2008). The possibility that intracellular protein had been biotinylated was excluded by using the ERK1/2 (cytosolic) and NF-κB (nuclear) protein expression. These proteins were negative in the pellet fraction (Fig. 2B), thus confirming selective labeling of cell surface proteins. Moreover, the specificity of the pellet fraction was demonstrated by determining the transmembrane protein APLP2 (Fig. 2B).

3.2. 17β-estradiol regulates the membrane ERα expression

To assess the changes of ERα in the plasma membrane induced by 17β-estradiol, primary anterior pituitary cells were treated with this hormone for 5, 15 and 30 min, and endogenous ERα was immunostained and visualized by confocal microscopy.

Under basal conditions, specific staining for ERα was mainly observed in the nucleus and cytoplasm of anterior pituitary cells, where a punctuated fluorescence signal was obtained. After 5 min of 17β-estradiol treatment, in addition to the nuclear and cytoplasmic localization, a strong surface staining for ERα at the plasma membrane was found. This distribution of the receptor remained for 30 min of stimulus (Fig. 3A).

The localization of ERα in the plasma membrane was confirmed by confocal microscopy using concanavalin A, a lectin that specifically binds to the α-mannosyl saccharides expressed in the core structures of plasma membrane glycoproteins, as a plasma membrane marker. A circumferential staining pattern was observed in non-permeabilized anterior pituitary cells stained with concanavalin A. Co-localization of ERα, and concanavalin A was clearly observed when the cells were treated for 15 min with 17β-estradiol or PPT (Fig. 3B). These data suggest that the ERα is expressed in

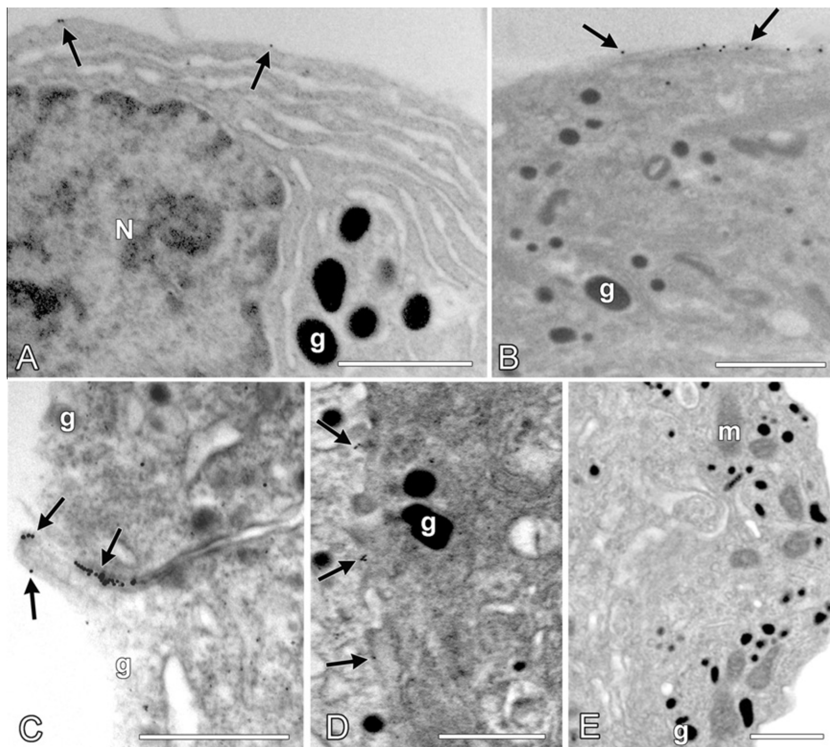

Fig. 1. Electron micrograph of lactotroph cells from primary culture immunolabeled for ERα by intact cell technique. (A) Lactotroph cell from control conditions, easily identified by pleomorphic granules stored in the cytoplasm (500–900 nm in diameter) with a few colloidal gold particles attached at the plasma membrane (arrows). (B–D) After 17β-estradiol treatment for 15 min, the number of gold particles increased significantly. (E) No immunolabeling was found when pre-absorption of the primary antibody with purified antigen was assayed. The images correspond to a representative experiment from a total of three with similar results. N, nucleus; g, granules; m, mitochondrias. *Bar* = 1 μm.

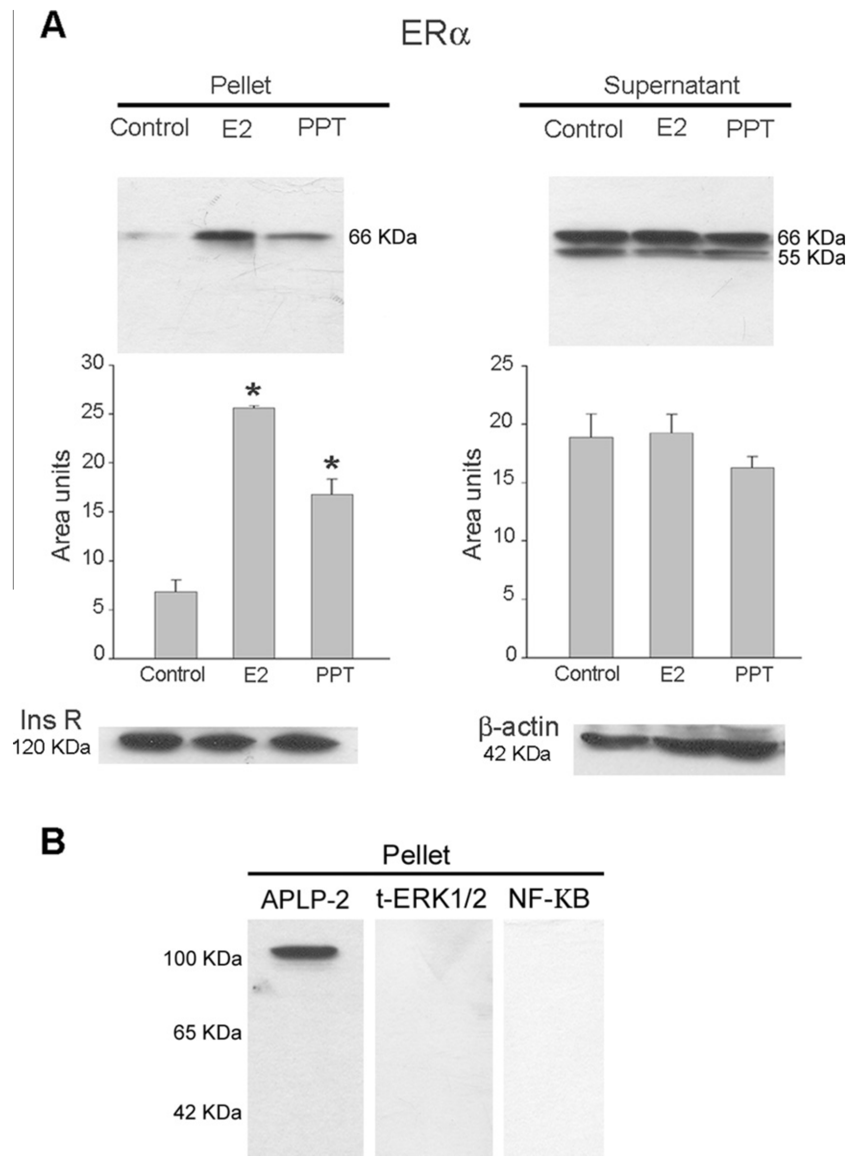

Fig. 2. Western blot analysis of cell-surface biotinylated proteins (pellet) and intracellular unbiotinylated proteins (supernatant) from anterior pituitary cells. (A) In the pellet fraction, the full-length of ERα is shown, which was increased by 17β-estradiol (E2) or PPT. The expression of insulin receptor (Ins R) and β-actin confirmed the equivalent total protein loading. Bars correspond to the full-length of ERα (≈66 kDa) and are shown as the means ± SEM of triplicate determinations from three independent experiments. $^*P < 0.001$ vs control. (B) The transmembrane protein APLP2 demonstrated the specificity of the pellet fraction. The cytoplasmic and nuclear proteins ERK1/2 and NF-κB were absent, indicating that intracellular proteins were not biotinylated. The bands correspond to a representative experiment from a total of three with similar results.

the plasma membrane and that this localization is regulated by 17β-estradiol.

To quantify the expression of ERα on the cell surface, flow cytometry analysis was performed. For basal conditions, 7.61 ± 0.8% of the total anterior pituitary cell populations expressed ERα on the cell surface, and 17β-estradiol treatment for 5, 15 and 30 min induced a significant increase in the geometrical mean of the ERα + fluorescence intensity ($P < 0.01$; Fig. 4A).

Considering that our previous results had shown the participation of membrane ERα in PRL secretion and cell proliferation (Gutiérrez et al., 2008), the ERα expression on the surface of this cell type was quantified. Under basal conditions, an anterior pituitary cell population (68.62% of all the scanned cells) was assigned as lactotrophs with a PRL antibody. Simultaneous labeling with a specific ERα antibody indicated that 7.7 ± 0.6% of these cells also expressed ERα at the cell surface. After 5, 15 and 30 min of 17β-estradiol treatment, a significant increase in the geometrical mean of ERα + fluorescence intensity on the surface of lactotroph cells was observed ($P < 0.01$; Fig. 4B). These results confirm the presence of ERα at the cell surface of anterior pituitary cells and indicate that 17β-estradiol induced a rapid redistribution of endogenous ERα, suggesting the existence of ERα traffic to the membrane.

3.3. PKCα and ERK1/2 are activated by 17 β-estradiol and ERα selective agonist

Considering that PKCα translocation to the cell membrane is evidence of its activation, we studied the spatial–temporal distribution of PKCα after 17β-estradiol or PPT stimulation by immunofluorescence. Under basal conditions, the distribution of PKCα in anterior pituitary cells was predominantly localized in the cytosol,

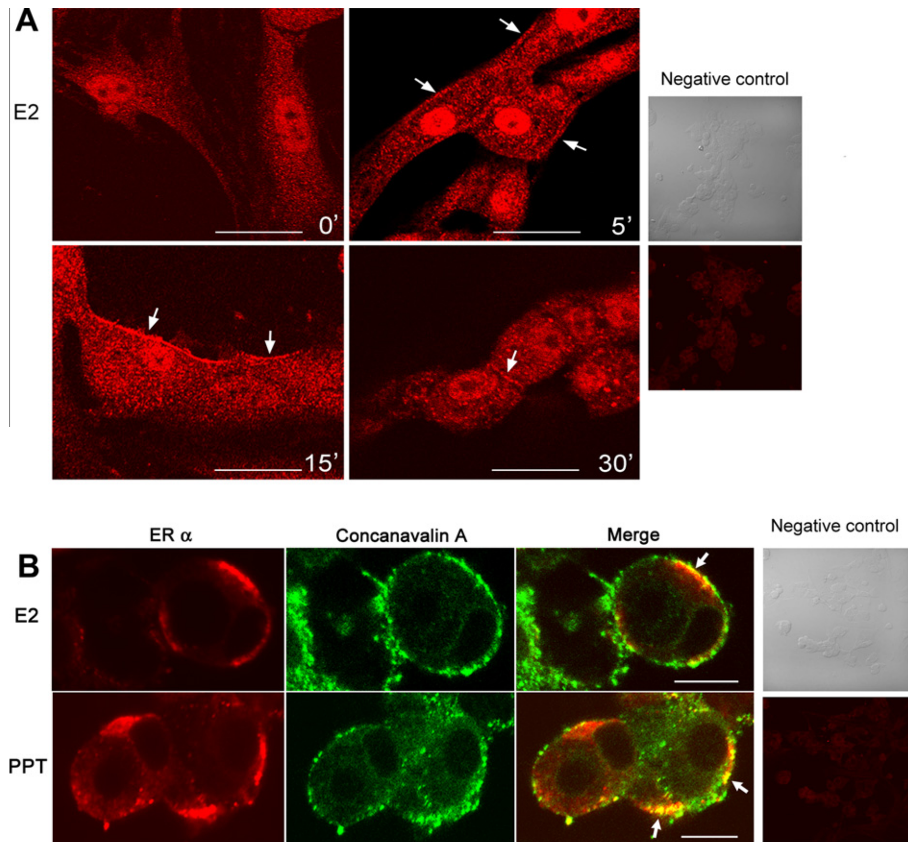

Fig. 3. Immunolabeling of ERα in anterior pituitary cells treated with E2 or PPT. (A) In estrogen-free conditions (0 min), ERα was found in the nucleus and cytoplasm of anterior pituitary cells. However, after E2 exposition for 5, 15 and 30 min, ERα was also detected in the cellular periphery (arrows). (B) In non-permeabilized anterior pituitary cells treated with or PPT E2 for 15 min, co-localization of ERα (red) and Concanavalin A (green) was observed (arrows), suggesting the presence of ERα in the plasma membrane. No immunofluorescence was found when the primary antibody was replaced by 1% PBS–BSA (Negative control). The images correspond to a representative experiment from a total of three with similar results. *Bar* = 20 μm.

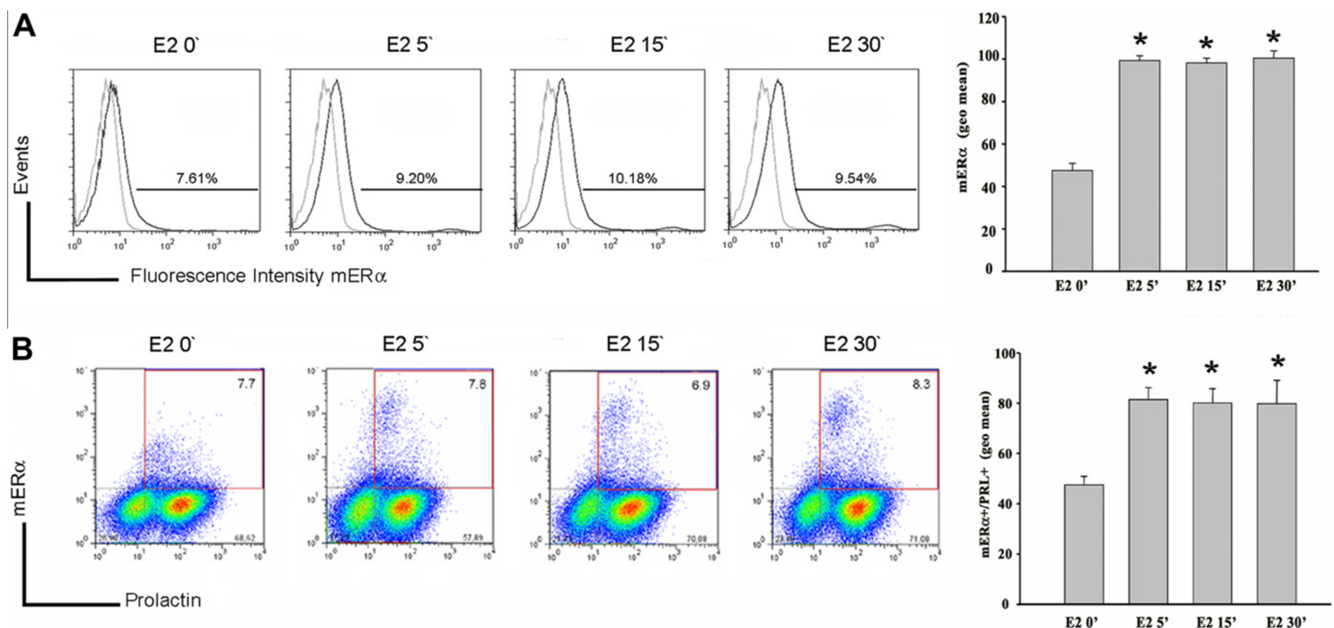

Fig. 4. Identification of ERα on the cell surface of total anterior pituitary cells by flow cytometry. (A) In control, 7.61 ± 0.8% of anterior pituitary cells showed ERα expression on the cell surface and after E2 stimulation for 5, 15 and 30 min with a significant increase in the geometrical mean of ERα + fluorescence intensity in the plasma membrane being observed (10^5 events for each experimental condition, $^*P < 0.01$ vs E2 0′). (B) Approximately the 70% of the total anterior pituitary cells were immunolabeled for prolactin and assigned as lactotrophs. In basal conditions, 7.7 ± 0.6% of this cell population expressed ERα in the membrane (mERα). After E2 stimulation for 5, 15 and 30 min, this population showed a significant increase in the geometrical mean of ERα + fluorescence intensity in the plasmatic membrane compared to control. Similar results were obtained in 3 independent experiments (10^5 events for each experimental condition, $^*P < 0.01$ vs E2 0′).

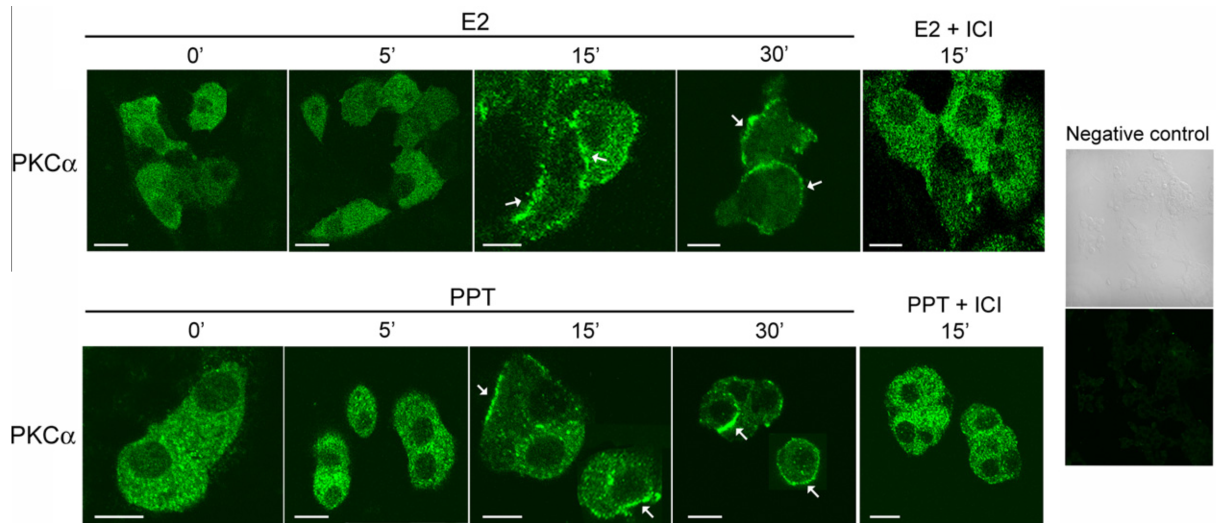

Fig. 5. Immunostaining of PKCα in anterior pituitary cells treated with E2 or PPT for 5, 15 and 30 min. Translocation of PKCα to the plasma membrane (arrows) was observed after 15 min of treatment with E2 or PPT with this mobilization being inhibited by ICI 182780. The images correspond to a representative experiment from a total of three with similar results. *Bar* = 20 μm.

with the localization of PKCα remaining unaltered during the first 5 min of stimulation. However, the treatments for 15 or 30 min led to a significant distribution shift of PKCα from throughout the cytosol to primarily the cell periphery. Pretreatment of anterior pituitary cells with ICI for 30 min prevented PKCα translocation in response to 17β-estradiol or PPT for 15 min (Fig. 5).

The Western blot analysis showed that 17β-estradiol increased the ERK1/2 phosphorylation in a time-dependent manner, starting after 5 min with a peak at 15 min of stimulation. This activation persisted up to 30 min only in cultures treated with 17β-estradiol. Following PPT stimulation, a rapid increase in ERK1/2 phosphorylation was detected, reaching maximum phosphorylation at 5 min and remaining for 15 min. We also tested the ability of the ER antagonist ICI to block hormone induced-ERK activation. Again, ERK phosphorylation was increased after 15 min stimulation with 17β-estradiol or PPT, and pre-treatment with ICI completely blocked this effect (Fig. 6).

The activation of PKCα and ERK1/2 was shown to be mediated by the membrane ER, by using an estradiol covalently linked to membrane-impermeable BSA, which retained its ability to induce rapid extranuclear-mediated nongenomic signaling. Stimulation with this estradiol conjugated for 15 min induced PKCα translocation to the plasma membrane (Fig. 7A) and ERK1/2 phosphorylation (Fig. 7B) with both these effects being reversed by ICI 182780.

We investigated the contribution of PKC in the ERK1/2 phosphorylation using Gö6976 (10 μM). This PKC inhibitor reversed the ERK1/2 phosphorylation induced by 17β-estradiol applied for 15 min (Fig. 8A), thereby suggesting that PKC is up-stream of ERK1/2 and is required to phosphorylate ERK1/2 under our experimental conditions.

Finally, with the aim of elucidating if PKCα is involved in the translocation mechanism of ERα, anterior pituitary cells were pre-incubated with a range of concentrations of the calcium-dependent PKC inhibitor Gö6976 (1, 5 and 10 μM) for 30 min, and then stimulated with 17β-estradiol for another 15 min. All doses tested were unable to affect the increase of ERα expression at the plasma membrane induced by 17β-estradiol, and the geometrical mean of ERα + fluorescence intensity in lactotroph cells did not change with the addition of the PKC inhibitor. These results suggest that PKCα is not involved in the mechanism of ERα translocation. The higher dose used of this inhibitor is shown as being representative (Fig. 8B).

4. Discussion

In the present study, we have demonstrated the presence of ERα in the cell surface of anterior pituitary cells, showing that 17β-estradiol was able to modulate this subcellular localization within a short time of stimulation. In pituitary tumoral GH3/B6/F10 cells, ERα was visualized by immunofluorescence, suggesting its association with the plasma membrane (Norfleet et al., 1999, 2000). In addition, the presence of the membrane ER has been indirectly suggested by rapid activation of different signaling pathways (Aguilar et al., 2006; Bulayeva et al., 2004, 2005). However, there is no good evidence that ERα spans the membrane or contains an extracellular domain. In relation with this topic, our results showed ERα on the surface of lactotrophs by ultrastructural-immuno-staining from primary anterior pituitary cell cultures. Taking into account that this technique is performed on living cells, where the primary antibody is in contact with the cell surface, it is very likely that the membrane ER has an extracellular region. Furthermore, our results with flow cytometry achieved in living dispersed cells support this idea. Previous investigations carried out on other tissues have identified ERα on the cell surface of both neurons (Dominguez and Micevych, 2010; Gorosito et al., 2008) and glial cells (Bondar et al., 2009) in the hypothalamus by biotinylation and in blood lymphocytes by flow cytometry (Pierdominici et al., 2010). Also, several studies using membrane-impermeable estrogen, such as estradiol-BSA or estradiol-peroxidase, have demonstrated the rapid effects of steroid hormones initiated in the plasma membrane (Bulayeva et al., 2004; Gorosito and Cambiasso, 2008; Gutiérrez et al., 2008; Zárate et al., 2009; Zhang et al., 2010). One of the most controversial issues regarding the membrane ER is related to its association with the plasma membrane, because the classic ER lacks a hydrophobic amino acid sequence for insertion into the plasmalemma. However, using hydropathicity analysis, the presence of the potential transmembrane domain near the NH2 terminal of classical ERα has recently been suggested (Bondar et al., 2009).

In this investigation, we used an antibody directed to the C-terminal of the classic intracellular ERα, suggesting that the membrane ERα is the same, or very similar to, that of the intracellular receptor. It has previously been reported that cells derived from combined ERα/β knockout mice lack any estrogen binding protein

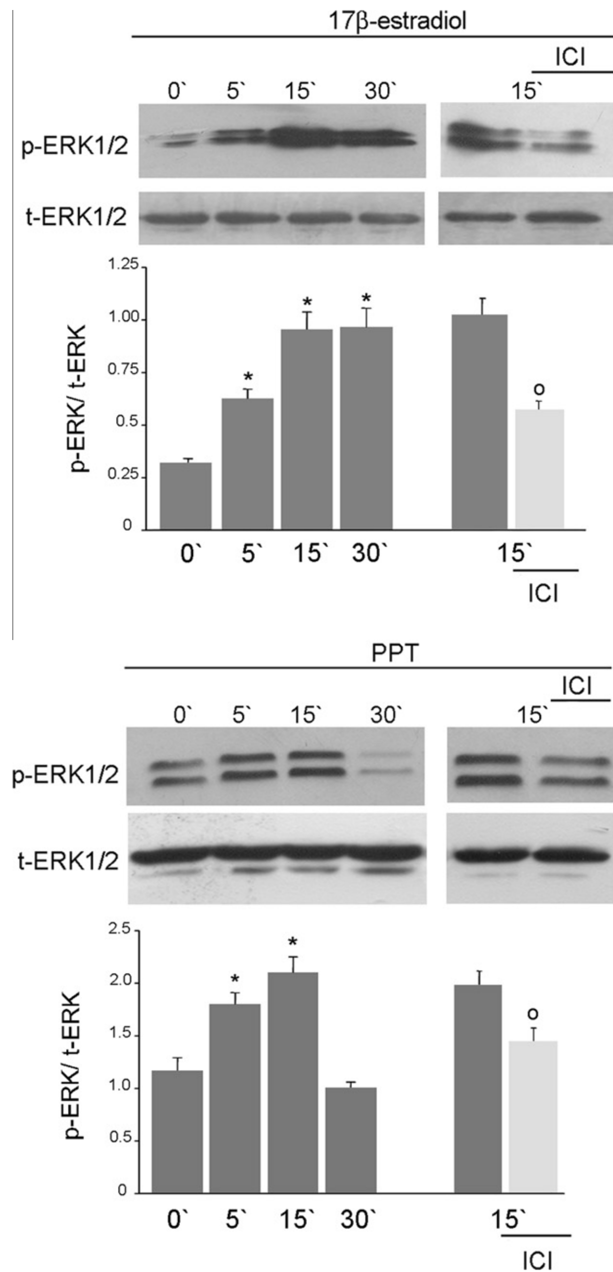

Fig. 6. Effect of E2 and the ERα agonist PPT on ERK1/2 phosphorylation. E2 or PPT stimulated ERK1/2 phosphorylation, starting from 5 min, with this effect being inhibited by the pre-treatment with 100 nM of ICI 182780 for 30 min. The bands correspond to a representative experiment. The p-ERK/t-ERK ratio was quantified and the values are shown as means ± SEM for three independent experiments. $^{*}P < 0.01$ vs E2 0′; $^{\circ}P < 0.01$ vs E2 15′.

(including at the plasma membrane) or the ability to activate signaling pathways to mediate the biological effects of estrogens on the cell (Pedram et al., 2006).

It is generally accepted that target tissue responsiveness to 17β-estradiol is principally controlled by the amount and type of ER (Friend et al., 1995; Taylor et al., 2010). We detected two proteins of different molecular masses in the supernatant fraction, namely the full-length ERα (66 kDa) and a smaller ER protein (50–55 kDa). The protein of 50–55 kDa was previously reported in the pituitary extracts from rat pituitary cells (Geffroy-Roisne et al., 1992), but its functional significance is still unclear. Related to this, a protein of 53 kDa was identified during the ontogeny of pituitary gland (Pasqualini et al., 1999), suggesting that it may play a physiological role in the rat pituitary development (Pasqualini et al., 2001). Concerning with the molecular mass of ERα present in the plasma membrane, different reports have shown varying results. In hypothalamic astrocytes and neurons, the full-length (66 kDa) and a smaller 52 kDa of ERα were detected in the plasma membrane (Bondar et al., 2009; Dominguez and Micevych, 2010), while a unique ERα band of 55 kDa was identified in the cell surface of hypothalamic neurons (Gorosito et al., 2008). Our results showed only the presence of the full-length ERα (66 kDa) in the plasma membrane, suggesting that it is the major ERα protein in the anterior pituitary cell surface.

The regulation of subcellular localization of different subtypes of ER is a mechanism by which signaling pathways can be differentially activated to promote both rapid as well as genomic actions (Evinger and Levin, 2005; Smith and O'Malley, 2004). The modulation of membrane ERα by estradiol has been described in neurons (Gorosito et al., 2008) and astrocytes (Bondar et al., 2009). In the present work, we demonstrated that 17β-estradiol stimulus induced an increase in the ERα expression in the plasma membrane of anterior pituitary cells, with a time course very similar to that previously reported in neurons (Dominguez and Micevych, 2010). In our study, the rapid increase of ERα expression observed in the plasma membrane (⩽15 min of stimulus) may indicate that *de novo* synthesis is not occurring, suggesting that 17β-estradiol stimulates the translocation of endogenous ERα at the plasma membrane.

The effects of 17β-estradiol on lactotroph cells have been studied in our laboratory, demonstrating different actions such as stimulating the PRL secretion and modulating the cell proliferation (Gutiérrez et al., 2005, 2010). These varied actions of estradiol can be mediated, at least in part, by the activation of the membrane pools of ER (Gutiérrez et al., 2008; Zárate et al., 2009). To elucidate the variations of membrane ERα expression induced by estradiol in lactotrophs, we quantified its fluorescence intensity by flow cytometry. Our results showed that about 8% of lactotrophs expressed ERα at the cell surface, increasing the fluorescence intensity after hormone treatment. This rapid increase in ERα expression strengthens the idea of translocation of intracellular ERα to the plasma membrane. It is possible that this subpopulation of lactotrophs that express membrane ERα could exert a regulatory role on lactotroph population, responding rapidly to hormonal demand by releasing the prolactin stored in the granules, until estradiol through the genomic pathway stimulates prolactin synthesis. In a previous work, we have shown that 17β-estradiol was able to induce a fast prolactin release, showing lactotrophs with morphological features indicative of a significant activation of hormone release, characterized by scarce mature secretory granules in the cytoplasm and exocytosis figures (Gutiérrez et al., 2008). Moreover, it has been demonstrated that the rapid exocytosis of PRL is regulated by membrane ERα signaling to specific Ca(2+) channels in GH3B6 (Bulayeva et al., 2005).

Our results showed a weak expression of ERα at the anterior pituitary cell surface, in agreement with publications showing that approximately 5–10% of total ER is found at the plasma membrane in many cells, which may include both ERα and ERβ, depending on the cell type (Levin, 2009a). Considering our previous results that showed the presence of the membrane ERα and the absence of the β subtype of ER in lactotroph cells (Gutiérrez et al., 2008), we postulate that the α subtype could be the main mediator of the estradiol effects exerted at the plasma membrane of anterior pituitary cells.

The mechanism of translocation of ER to the plasma membrane is still not fully understood. Serine 522 has been identified to be necessary for ER translocation, promoting interaction with the caveolin-1 protein (Razandi et al., 2003). Also, cysteine 447 is a site for palmitoylation that promotes the association of ER with caveo-

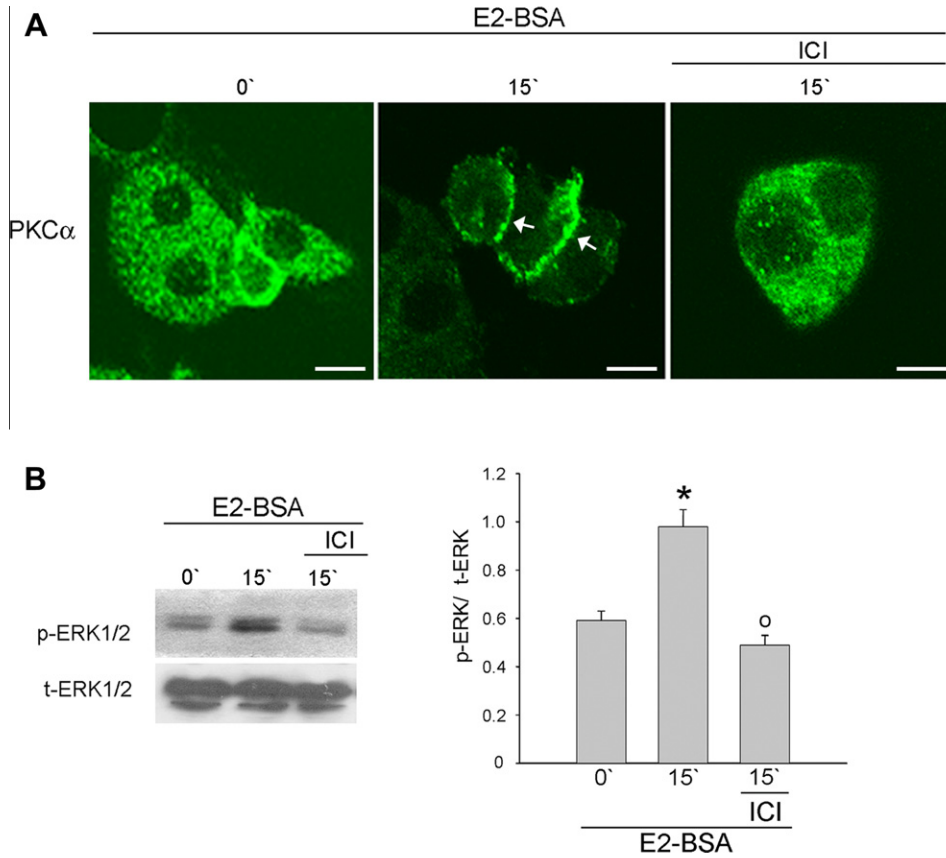

Fig. 7. Effect of estradiol-BSA on PKCα translocation and ERK1/2 phosphorylation. This membrane-impermeable conjugated estradiol-BSA stimulated the PKCα translocation to the plasma membrane (arrows) (A) and ERK1/2 phosphorylation (B), with these effects being reversed by the inhibitor ICI 182780. The bands correspond to a representative experiment. The p-ERK/t-ERK ratio was quantified and the values are shown as means ± SEM for three independent experiments. $^{*}P < 0.01$ vs estradiol-BSA 0′; $^{o}P < 0.01$ vs estradiol-BSA 15′. *Bar* = 20 μm.

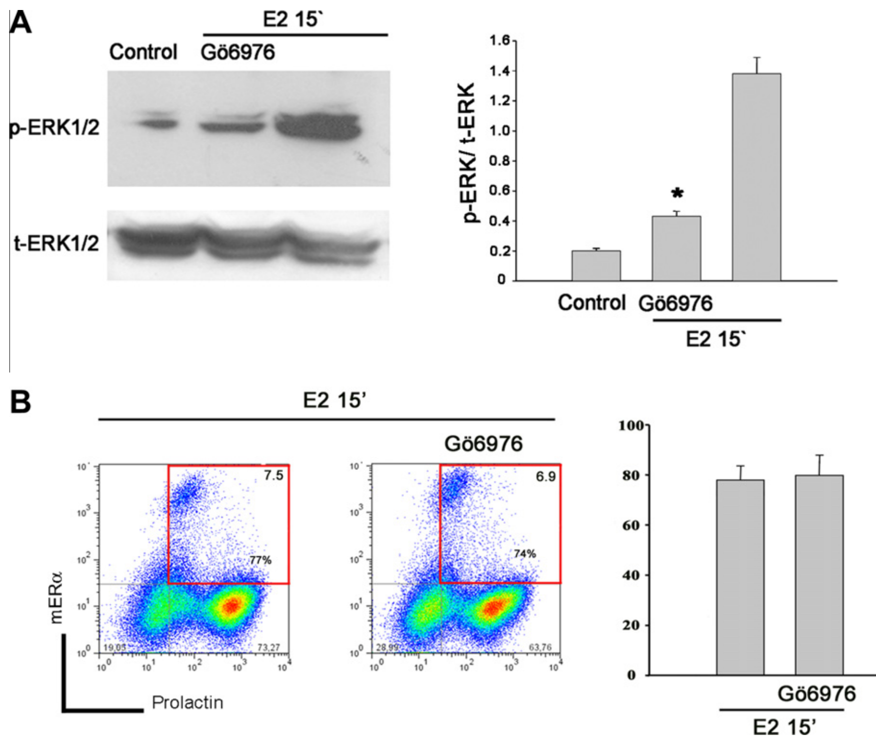

Fig. 8. Effect of Gö6976, a calcium-dependent PKC inhibitor. (A) Gö6976 reversed the ERK1/2 phosphorylation induced by E2. The bands correspond to a representative experiment. The p-ERK/t-ERK ratio was quantified and the values are shown as means ± SEM for three independent experiments. $^{*}P < 0.01$ vs E2 15′. (B) Gö6976 was unable to affect the increase in the membrane ERα expression induced by E2 in lactotroph cells, with no significant changes in the geometrical mean of ERα + fluorescence intensity found between E2 vs E2 + Gö6976. Similar results were obtained in 3 independent experiments (10^5 events for each experimental condition).

lin-1 (Acconcia et al., 2004). Palmitoylation seems to be necessary for plasma membrane localization and rapid signaling through ERK and PI3 kinases to provoke cell proliferation. This takes place in proximity to the caveolae rafts, leading to calcium and cAMP generation, and also the activation of both proximal kinases (Src, PI3K) and distal kinases (ERK, AKT). In the present report, we have observed that 17β-estradiol induced a rapid activation of PKCα and phosphorylation of ERK. Interestingly, the time course for membrane recruitment of PKC by ER was closely related to that of the maximum (15 min) E2-induced ERK activation. These results are further evidence that the activation of the PKCα–MAPK pathway occurs through ER associated with the plasma membrane, suggesting the expression of a functional membrane ERα. Moreover, our results using PPT have shown that this selective ERα agonist was able to increase the ERα expression in the plasma membrane and to activate the PKCα/ERK 1/2 pathway, thus mimicking the actions of estradiol. These findings suggest that ligand-occupied ERα stimulates the translocation of endogenous ERα at the plasma membrane, and activates signaling pathways to induce a biological effect in normal anterior pituitary cells.

In summary, our results provide evidence for the presence of ERα in the plasma membrane of normal anterior pituitary cells, with this expression being increased by 17β-estradiol, suggesting that this steroid hormone stimulates the translocation of endogenous ERα to the plasma membrane. In the anterior pituitary gland, 17β-estradiol might modulate the different ER pools in a coordinated but poorly understood manner, finally leading to cellular biological effects.

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