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Estradiol increases cAMP in the oviductal secretory cells through a nongenomic mechanism

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Abstract

In the rat oviduct, estradiol (E_2) accelerates egg transport by a nongenomic action that requires previous conversion of E_2 to methoxyestrogens via catechol-*O*-methyltranferase (COMT) and activation of estrogen receptor (ER) with subsequent production of cAMP and inositol triphosphate (IP3). However, the role of the different oviductal cellular phenotypes on this E_2 nongenomic pathway remains undetermined. The aim of this study was to investigate the effect of E_2 on the levels of cAMP and IP3 in primary cultures of secretory and smooth muscle cells from rat oviducts and determine the mechanism by which E_2 increases cAMP in the secretory cells. In the secretory cells, E_2 increased cAMP but not IP3, while in the smooth muscle cells E_2 decreased cAMP and increased IP3. Suppression of protein synthesis by actinomycin D did not prevent the E_2-induced cAMP increase, but this was blocked by the ER antagonist ICI 182 780 and the inhibitors of COMT OR 486, G protein-α inhibitory ($G\alpha_i$) protein pertussis toxin and adenylyl cyclase (AC) SQ 22536. Expression of the mRNA for the enzymes that metabolizes estrogens, *Comt*, *Cyp1a1*, and *Cyp1b1* was found in the secretory cells, but this was not affected by E_2. Finally, confocal immunofluorescence analysis showed that E_2 induced colocalization between ESR1 (ERα) and $G\alpha_i$ in extranuclear regions of the secretory cells. We conclude that E_2 differentially regulates cAMP and IP3 in the secretory and smooth muscle cells of the rat oviduct. In the secretory cells, E_2 increases cAMP via a nongenomic action that requires activation of COMT and ER, coupling between ESR1 and $G\alpha_i$, and stimulation of AC.

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Introduction

In the female genital tract, estrogens regulate a variety of biological functions including gamete transport, oocyte fertilization, embryo development, and implantation (reviewed in Croxatto (1996)). The classical mechanism by which estradiol (E_2) affects its target cells comprises binding to estrogen receptors (ERs) and modification of gene expression and protein synthesis (Nilsson *et al.* 2001). However, this model cannot explain E_2 effects that are not blocked by inhibitors of transcription or translation, or that are too rapid to be due to changes in gene expression. These features do not appear compatible with the classical genomic actions and are termed nongenomic (Lössel & Wheling 2003, Lössel *et al.* 2003). Nongenomic actions of E_2 often involve activation of G protein-α inhibitory ($G\alpha_i$), stimulation of intracellular signal transduction pathways that include generation of second messengers such as cAMP and inositol triphosphate (IP3), and activation of protein kinase A (PKA) or phospholipase C (PLC) in the E_2-target cells (Nadal *et al.* 2001, Wyckoff *et al.* 2001, Acconcia *et al.* 2005, Hill *et al.* 2010). Recently, it has been also proposed that estrogen metabolites could be responsible for some E_2 nongenomic actions (Mueck & Seeger 2010, Rincón-Rodríguez *et al.* 2013).

In the rat, the duration of oviductal egg transport is dependent on ovarian hormones and mating-associated signals (for review see Croxatto (2002)). A single injection of E_2 on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 to <24 h (Ortíz *et al.* 1979). We have previously demonstrated that RNA and protein synthesis inhibitors did not block E_2-induced acceleration of oviductal egg transport in unmated rats, indicating that E_2 accelerates oviductal egg transport by a nongenomic mechanism (Orihuela *et al.* 2001). This E_2 nongenomic pathway involves a previous conversion of E_2 to methoxyestradiols, mediated by the enzyme catechol-*O*-methyltransferase (COMT; Parada-Bustamante *et al.* 2007, 2010), activation of ER and adenylyl cyclase (AC) (Orihuela *et al.* 2003), and successive production of cAMP and IP3 (Orihuela *et al.* 2003, 2006, 2013).

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The rat oviduct is a tubular organ mainly composed of an intrinsic layer smooth muscle fiber, the myosalpinx, and an innermost highly folded mucosa (secretory, ciliated, and stromal cells), the endosalpinx. From the ovary to the uterus, it is possible to distinguish in the oviduct the fimbria, the ampulla, the isthmus, and the utero-tubal junction. In the ampulla, ciliated cells are more abundant whereas in the isthmus secretory cells are predominant (Croxatto 1996). Transport of oocytes through the ampulla depends mainly on the activity of ciliated cells whose cilia beat toward the uterus. In contrast, egg transport through the isthmus depends on the contractile activity of the smooth muscle cells (Moore & Croxatto 1988*a*, Ríos *et al*. 2007). The actual vision on the mechanics involved in the acceleration of E_2-induced egg transport in the rat oviduct indicate that E_2 induces the release of paracrine signals from the secretory cells initiating waves of myosalpinx (mainly composed of smooth muscle cells) contractions that lead to increase in the speed of the egg transport (Moore & Croxatto 1988*b*, Croxatto 2002, Parada-Bustamante *et al*. 2012). Therefore in this work, we examined the contribution of the secretory and smooth muscles cells in the E_2 nongenomic signaling that accelerates egg transport in the rat oviduct. First, we determined the effect of E_2 on the levels of cAMP or IP3 in the secretory and smooth muscle cells from rat oviducts. The results oriented us to investigate the mechanism by which E_2 increases cAMP in the secretory cells. Thus, we examined the effect of E_2 on the cAMP level in the secretory cells under conditions in which protein synthesis, ER, COMT, $G\alpha_i$, or AC activity was quenched by selective inhibitors. In addition, the effect of E_2 on the expression of some enzymes involved in estrogen metabolism and colocalization between ESR1 (ERα) and the $G\alpha_i$ protein was determined in the oviductal secretory cells.

Materials and methods

Animals

Locally bred Sprague–Dawley rats weighing 200–260 g were used. The animals were kept under controlled temperature (21–24 °C), and lights were on from 0700 to 2100 h. Water and pelleted rat chows were supplied *ad libitum*. Female mature rats were used in the estrus stage. The phases of the estrous cycle were determined by daily vaginal smears (Turner 1961) and all females were used after showing two consecutive 4-day cycles. The care and manipulation of the animals was done in accordance with the ethical guidelines of the Universidad de Santiago de Chile.

Culture of primary cells from rat oviducts

Secretory cells

A protocol to obtain secretory cells instead of ciliated cells was performed in this work (Morales *et al*. 2000, 2006). For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hanks' solution (Sigma Chemical Co.) of pH 7.4. The whole oviduct was cut into small (4–8 mm^2) pieces in Hanks' solution and then the epithelial cells were mechanically removed from the rest of the tissue. The cell suspension was centrifuged at 800 ***g*** during 5 min, washed, and seeded into six-well tissue culture plates coated with collagen I, rat-tail (Invitrogen) in DMEM/high modified medium with 4.0 mM L-glutamine and 4.500 mg/l glucose free of Phenol Red (cat. no. SH30284.02, HyClone, Thermo Scientific, Walthman, MA, USA) supplemented with 10% (V/V) of fetal bovine serum (Cat. No. SH30396.03, HyClone), sodium pyruvate 1 mM, and antibiotics: 100 UI/ml of penicillin and 100 μg/ml of streptomycin. The epithelial cells were incubated at 37 °C in an atmosphere of 5% (vol/vol) CO_2 for at least 3 days to 75–80% confluence and characterized by immunofluorescence staining with a cytokeratine antibody. The presence of secretory and ciliated cells in the primary cultures was determined using immunohistochemical staining for MUC1 (secretory cell marker; DeSouza *et al*. 1998) and β-tubulin IV (ciliated cell marker; Shao *et al*. 2007, Nutu *et al*. 2009).

Smooth muscle cells

For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hanks' solution (Sigma Chemical Co.) of pH 7.4. The whole oviduct was cut into small (4–8 mm^2) pieces in Hanks' solution and then the smooth muscle cells were mechanically removed from the rest of the tissue and treated with collagenase, type I (Invitrogen) for 1 h to further the disaggregation of the cells. The cell suspension was centrifuged at 1200 ***g*** during 5 min, washed, and seeded into six-well tissue culture plates (Becton Dickinson & Co., Franklin Lakes, NJ, USA) in DMEM/high modified medium with 4.0 mM L-glutamine and 4.500 mg/l glucose free of Phenol Red (cat. no. SH30284.02, HyClone, Thermo Scientific) supplemented with 10% (vol/vol) of fetal bovine serum (cat. no. SH30396.03, HyClone), sodium pyruvate 1 mM, and antibiotics: 100 UI/ml of penicillin and 100 μg/ml of streptomycin. Smooth muscle cells were incubated at 37 °C in an atmosphere of 5% (vol/vol) CO_2 for at least 7 days to reach 75–80% confluence and characterized by immunohistochemical staining with an α-actin antibody.

Treatments

Primary cultures of secretory and smooth muscle cells were treated with 10^{-9} M of E_2 (Sigma) or ethanol 0.01% as vehicle. Other primary cultures of secretory cells were also incubated with the protein synthesis inhibitor actinomycin D (ActD, 1 μg/μl, Sigma), the ER antagonist ICI 182 780 (25 μg/μl, Tocris Bioscience, Bristol, UK), the AC inhibitor SQ 22536 (7.5 μg/μl, Calbiochem, La Jolla, CA, USA), the selective COMT activity inhibitor OR 486 (25 μg/μl, Tocris Bioscience), or the $G\alpha_i$ protein inhibitor pertussis toxin (PTX, 1 μg/μl) as appropriate to each experiment. DMSO 0.01% was used as a vehicle of the inhibitors as it is more efficient than ethanol to dissolve nonpolar or semi-polar drugs.

Measurement of cAMP levels

The primary cell cultures were sonicated in 100 μl of ice cold 10% (v/v) trichloroacetic acid (TCA) and centrifuged for 15 min

at 5000 ***g*** at 4 °C. The pellet was discarded and the supernatant was washed four times with five volumes of water-saturated diethyl ether. The upper layer was discarded after each wash. Following the last wash, the aqueous extract was dried under a stream of nitrogen at 60 °C. The levels of cAMP in dried extracts were determined using Biotrak cAMP enzyme immunoassay system, cat. no. RPN 225 (Amersham Pharmacia Biotech). This kit is based on competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. This allows the construction of a standard curve and the measurement of cAMP levels in unknown samples. 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide was used as a substrate for color development. The optical density was read at 630 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Measurement of IP3 levels

The primary cell cultures were sonicated in 100 µl of ice-cold 1 M TCA and centrifuged for 10 min at 1000 ***g*** at 4 °C. The pellet was discarded and the supernatant was incubated for 15 min at room temperature. TCA was removed from the supernatant with 0.5 ml of a solution 1,1,2-trichloro-trifluoro-ethane (Sigma Chemical Co.)–trioctylamine (Sigma), 3:1 (v/v). The levels of IP3 were determined using the Inositol-1,4,5-trisphosphate [^{3}H] Radioreceptor Assay Kit, cat. no. NEK064 (NEN Life Science Products, Boston, MA, USA). This kit is based on a competition between non-radioactive IP3 and a fixed quantity of [^{3}H]-IP3 for a limited number of calf cerebellum IP3 receptor-binding sites. This allows the construction of a standard curve and the measurement of IP3 levels in unknown samples.

Real-time PCR

Total RNA from primary secretory cell cultures was isolated using TRIzol Reagent (Invitrogen). One microgram of total RNA of each sample was treated with DNase I, amplification grade (Invitrogen). The single-strand cDNA was synthesized by RT using the superscript III reverse transcriptase first-strand system for RT-PCR (Invitrogen), according to the manufacturer's protocol. The Light Cycler Instrument (Roche Diagnostics GmbH) was used to quantify the relative gene expression of *Comt*, *Cyp1a1*, and *Cyp1b1* in the oviductal secretory cells; *Gapdh* was chosen as the housekeeping gene for load control. The SYBR Green I double-strand DNA binding dye (Roche Diagnostics) was the reagent of choice for these assays. Primers for *Comt*, 5′-CAC CTA CTG CAC ACA GAA GG-3′ (sense) and 5′-GTT AGT GTG TGC ACT CGA AGC-3′ (antisense); *Cyp1a1*, 5′-AGT TTG GGG GAG GTT ACT GGT TC-3′ (sense) and 5′-GGA CAT CAC AGA CAG CCT CAT T-3′ (antisense); *Cyp1b1*, 5′-CCT TGG GGA CTC TCA GGT TG-3′ (sense) and 5′-CCA TTC TTC TGC TAC TCG TTT CG-3′; and *Gapdh*, 5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (anti sense). All real-time PCR assays were carried out in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 25 min, followed by 40 cycles of denaturizing and annealing–amplification (95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s), and finally one cycle of melting (95–60 °C). To verify specificity of the product, amplified products were subject to melting curve analysis as well as electrophoresis, and product sequencing was carried out to confirm identity using an ABI Prism310 Sequencer. The expression of *Comt*, *Cyp1a1*, and *Cyp1b1* was determined using the equation: $Y=2^{-\Delta Cp}$ where Y is the relative expression, Cp (crossing point) is the cycle in the amplification reaction in which fluorescence begins to be exponential above the background base line, $-\Delta Cp$ is the result of subtracting Cp value of *Cyp1a1*, *Cyp1b1*, and *Comt* from Cp value of *Gapdh* for each sample. To simplify the presentation of the data, the relative expression values were multiplied by 10^3 (Livak & Schmittgen 2001).

Immunofluorescence

Oviductal cells were fixed in cold 4% paraformaldehyde in PBS of pH 7.4–7.6 for 2 h, transferred to 10% w/v sucrose in PBS for 60 min at 4 °C and 30% w/v sucrose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS–BSA for 120 min and incubated with mouse anti-cytokeratin (Santa Cruz Biotechnology, Inc.) or mouse anti-α-actin (Santa Cruz Biotechnology, Inc.) antibody 1:250 and 1:500 respectively. After washing with PBS, the preparations were incubated for 2 h with rabbit anti-mouse IgG FITC conjugate (Santa Cruz Biotechnology, Inc.) diluted 1:1000. The sections were washed and counterstained with 1 µg/ml of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA), washed again and then mounted in Fluoromount G. As negative controls, the primary antibody was replaced by preimmune serum. All sections were visualized under an Optiphot Epifluoresence Microscope (Olympus).

Immunohistochemical

Oviductal cells were fixed in cold 4% paraformaldehyde in PBS of pH 7.4–7.6 for 2 h, transferred to 10% w/v sucrose in PBS for 60 min at 4 °C and 30% w/v sucrose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS–BSA for 120 min and incubated with rabbit anti-MUC1 (Abcam, Cambridge, MA, USA) or mouse anti-β-tubulin IV (Sigma) antibody 1:100. After washing with PBS, the preparations were incubated for 2 h with goat anti-rabbit or anti-mouse IgG alkaline phosphatase conjugate (Chemicon International, Temecula, CA, USA). The alkaline phosphatase activity was detected by color development during incubation of the cells in 100 mM Tris/HCl of pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$, containing BCIP/NBT tablets (Sigma Chemical Co.; one tablet in 10 ml). As negative controls, the primary antibody was replaced by preimmune serum. All sections were visualized under an phase-contrast Optiphot Epifluoresence Microscope (Olympus).

Confocal microscopy

Oviductal cells were fixed in cold 4% paraformaldehyde in PBS of pH 7.4–7.6 for 2 h and transferred to 10% w/v sucrose in PBS for 60 min at 4 °C and 30% w/v sucrose in PBS at 4 °C

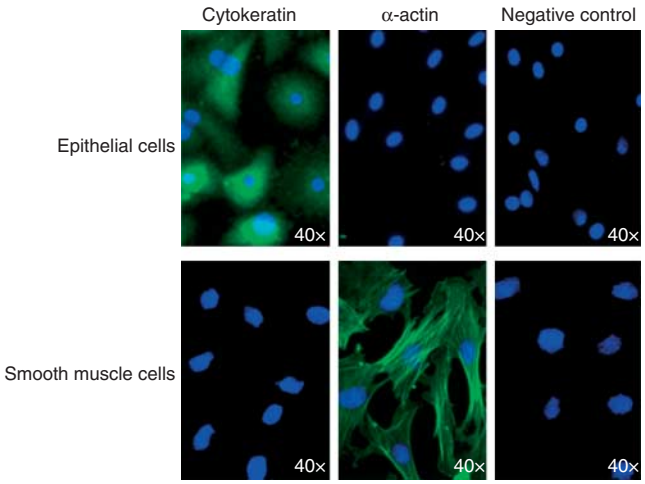

Figure 1 Expression of cytokeratin and α-actin in the primary cultures of epithelial and smooth muscle cells from rat oviducts. Representative photomicrographs of primary cultures of epithelial and smooth muscle cells from rat oviducts were processed by immunofluorescence microscopy to detect the expression of cytokeratin and α-actin. Note that cytokeratin (green) and α-actin (green) were only expressed in epithelial and smooth muscle cells respectively. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum.

overnight. Then, they were blocked with 1% PBS–BSA for 120 min and incubated with rabbit anti-ESR1 (Santa Cruz Biotechnology, Inc.) or mouse anti-$G\alpha_i$ (Santa Cruz Biotechnology, Inc.) antibody 1:50 in 1% PBS–BSA in a humidified chamber overnight. Followed by three rinses in PBS, the cells were incubated for 60 min at room temperature with secondary antibody Alexa Fluor 555-conjugated goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 588-conjugated goat anti-mouse IgG (Invitrogen) diluted in 1% PBS–BSA. The samples were subsequently washed with PBS and mounted in DABCO (Sigma). As negative controls, the primary antibody was replaced by preimmune serum. All sections were visualized with laser scanning confocal microscopy on a Axiovert 100 M microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

Data for cAMP and IP3 assays or real-time PCR from cultured oviductal cells were replicated five times for each treatment (for each culture experiment, oviductal cells were recovered from a pool of six different rats). Results subjected to statistical analysis were expressed as mean±S.E.M. Data were subjected to Kruskal–Wallis test, followed by Mann–Whitney *U* test for pairwise comparisons when overall significance was detected. Significance was accepted at $P<0.05$.

Results

E_2 differentially regulates cAMP and IP3 levels in secretory and smooth muscle cells of the rat oviduct

This experiment was designed to determine the effect of E_2 on the level of cAMP and IP3 in the oviductal secretory and smooth muscle cells. First, we confirmed the purity of the cells by immunofluorescence staining of cytokeratin or α-actin (Fig. 1) and also established that the primary cultures of the epithelial cells were conformed by secretory cells (Fig. 2). Therefore, secretory or smooth muscle cells were treated with ethanol or E_2 during 0, 1, 3, or 6 h and processed to measure the concentration of cAMP or IP3 as described in the 'Materials and methods' section. Replicas of this experiment are indicated in Figs 3 and 4.

Figure 3 shows that in the secretory cells, the basal cAMP level ranged from 48.1±21.1 to 63.0±19.5 fmol/μg of protein and in the smooth muscle cells it ranged from 25.8±5.9 to 32.4±6.9 fmol/μg of protein. In the secretory cells, treatment with E_2 increased threefold the amount of cAMP at 3 and 6 h but not at 1 h. In the smooth muscle cells, E_2 decreased threefold the amount of cAMP level at 3 h with no effect at 1 and 6 h. On the other hand, Fig. 4 shows that E_2 did not affect the basal level of IP3 in the secretory cells (vehicle, 134±45–164±53 fmol/μg of protein and E_2, 93±55–117±044 fmol/μg of protein) while in the smooth muscle cells E_2 increased IP3 level to fourfold at 6 h (vehicle, 150±60 fmol/μg of protein and E_2, 800±130 fmol/μg of protein) without any effect at 1 h (vehicle, 190±70 fmol/μg of protein and E_2, 140±90 fmol/μg of protein) and 3 h (vehicle, 130±70 fmol/μg of protein and E_2, 104±90 fmol/μg of protein).

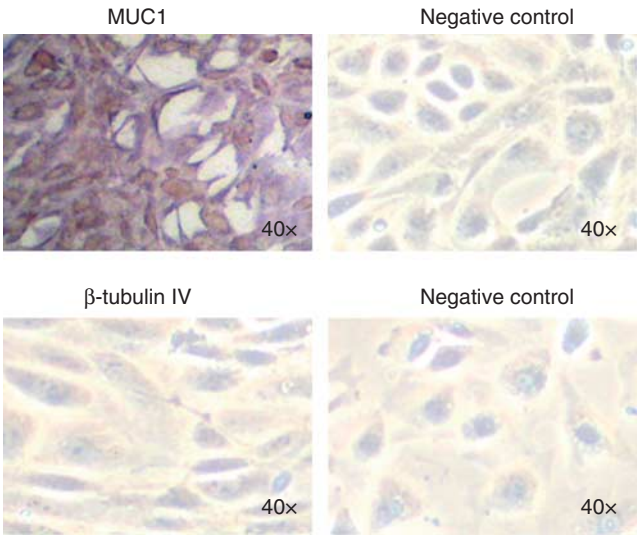

Figure 2 Primary cultures of epithelial cells from rat oviducts express MUC1 but not β-tubulin IV. Representative photomicrographs of primary cultures of epithelial cells from rat oviducts were processed by immunohistochemistry to detect the expression of MUC1 (marker for secretory cells) and β-tubulin IV (marker of ciliated cells). Note that positive immunoreactivity for MUC1 (purple) was only observed in the epithelial cells. Negative controls of the immunoreactivity were incubated with preimmune serum.

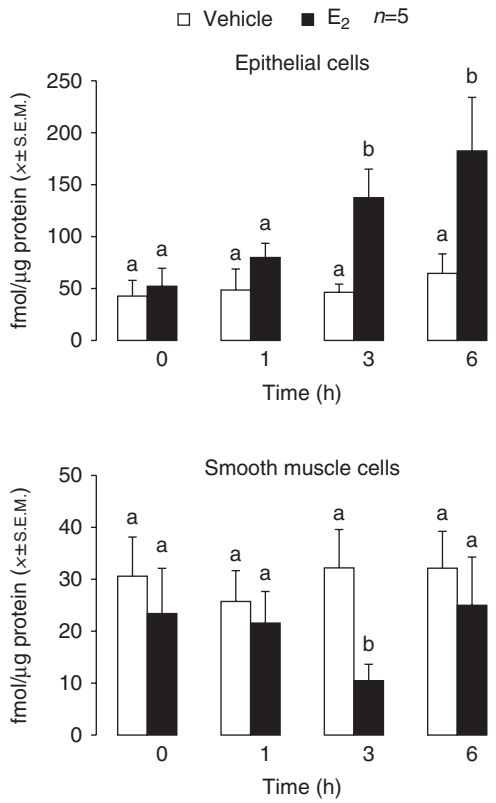

Figure 3 Effect of E_2 on the cAMP level in primary cultures of secretory and smooth muscle cells from rat oviducts. Primary cultures of secretory and smooth muscle cells from rat oviducts were treated with E_2 10^{-9} M and 0, 1, 3, or 6 h later the cAMP level was determined by an enzyme immunoassay system. Note that in the epithelial cells, E_2 increased the cAMP level 3 and 6 h after treatment, while in the smooth muscle cells E_2 decreased the cAMP level 6 h after treatment. This experiment consisted of five replicates. a≠b, $P<0.05$.

E_2 increased cAMP level by a nongenomic action in the oviductal secretory cells

This experiment was designed to determine whether the effect of E_2 on the cAMP level in the secretory cells occurs under conditions in which RNA and protein synthesis is suppressed. For this we used ActD, which is an inhibitor of RNA and protein synthesis. Primary cultures of secretory cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E_2 + DMSO, iii) ethanol + ActD, and iv) E_2 + ActD. At 3 h after treatment, cultured cells were processed to measure the concentration of cAMP as described in the 'Materials and methods' section. Replicas of this experiment are indicated in Fig. 5.

In the control group, the cAMP level was 58.3 ± 10.1 fmol/μg of protein, while in the E_2-treated group it was 283.9 ± 16.7 fmol/μg of protein. Administration of ActD alone or concomitant with E_2 neither affected the basal cAMP level (49.6 ± 12.3 fmol/μg of protein) nor the E_2-induced cAMP increase (230.3 ± 22.9 fmol/μg of protein) in the oviductal secretory cells.

E_2 increased cAMP level through activation of ER, $G\alpha_i$, and AC in the oviductal secretory cells

These experiments were carried out to determine whether the effect of E_2 on the cAMP level in the oviductal secretory cells is mediated by activation of ER, $G\alpha_i$, and AC. For this, we used ICI 182 780 that is an ER antagonist, PTX that is a highly specific inhibitor of heteromeric G proteins of the G_i class and SQ 22536 that is a selective inhibitor of AC activity. In each experiment, primary cultures of secretory cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E_2 + DMSO, iii) ethanol + inhibitor, and iv) E_2 + inhibitor. At 3 h after treatment, the cultured cells were processed to measure the concentration of cAMP as described in the 'Materials and methods' section. Replicas of this experiment are indicated in Fig. 6.

The results are shown in Fig. 6. In the control group, the cAMP level ranged from 51.1 ± 10.3 to 55.6 ± 17.1 pmol/μg of protein while in the E_2-treated group it ranged from 283.3 ± 19.7 to 316.9 ± 51.3 fmol/μg of protein. Administration of ICI 182 780, PTX, or SQ 22536 alone did not affect the basal cAMP level (range, 46.8 ± 19.2–64.2 ± 14.1 fmol/μg of protein) although

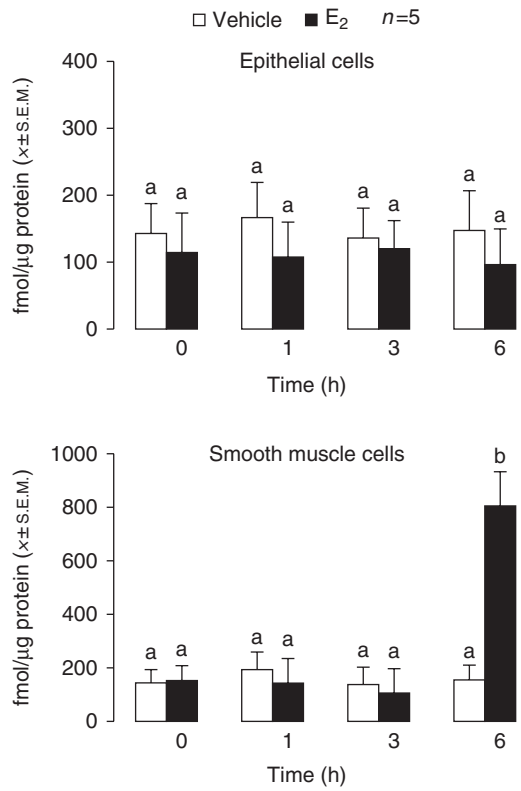

Figure 4 Effect of E_2 on the IP3 level in primary cultures of secretory and smooth muscle cells from rat oviducts. Primary cultures of secretory and smooth muscle cells from rat oviducts were treated with E_2 10^{-9} M and 0, 1, 3, or 6 h later the IP3 level was determined by a radioreceptor assay. Note that in the epithelial cells, E_2 did not affect the IP3 level while in the smooth muscle cells E_2 increased the IP3 level 6 h after treatment. This experiment consisted of five replicates. a≠b, $P<0.05$.

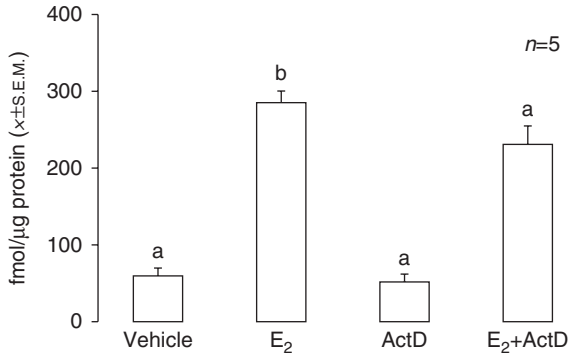

Figure 5 Effect of actinomycin D (ActD) on the E_2-induced cAMP increase in the oviductal secretory cells. Primary cultures of secretory cells from rat oviducts were treated with E_2 10^{-9} M alone or with the protein synthesis inhibitor ActD 1 µg/µl and 3 h later the cAMP level was determined by an enzyme immunoassay system. Note that ActD did not block the effect of E_2 on the cAMP level. This experiment consisted of five replicates. a≠b, $P<0.05$.

blocked the E_2-simulated cAMP increase (range, 83.4±21.5–57.5±9.9 fmol/µg of protein).

E_2 increased cAMP level through activation of COMT in the oviductal secretory cells

In this study, we investigated whether the effect of E_2 on the level of cAMP in the secretory cells occurs under condition in which COMT activity is suppressed. For this we used OR 486 which is a selective inhibitor of the COMT activity. Primary cultures of secretory cells from rat oviducts were divided into the following treatment groups: i) ethanol+DMSO; ii) E_2+DMSO; iii) ethanol+OR, 486; and iv) E_2+OR 486. At 3 h after treatment, the cultured cells were processed to measure the concentration of cAMP as described in the 'Materials and methods' section. Replicas of this experiment are indicated in Fig. 7A.

Figure 7A shows that in the control group, the cAMP level was 68.3±14.1 pmol/µg of protein, while in the E_2-treated group treated it was 243.9±36.7 fmol/µg of protein. Administration of OR 486 alone did not affect the basal cAMP level (71.8±23.4) although blocked the E_2-stimulated cAMP increase (69.5±19.4).

E_2 did not change the expression of the Comt, Cyp1a1, *and* Cyp1b1 *transcripts in the oviductal secretory cells*

As COMT and the cytochrome P450 isoforms CYP1A1 and CYP1B1 convert E_2 into methoxyestradiols, we determined the effect of E_2 on the mRNA levels for *Comt*, *Cyp1a1*, and *Cyp1b1*. Primary cultures of oviductal secretory cells were treated with ethanol or E_2 10^{-9} M and 3 h later the mRNA level for these enzymes was assessed by real-time PCR. Replicas of this experiment are indicated in Fig. 7B. E_2 treatment did not change the mRNA level of *Comt*, *Cyp1a1*, and *Cyp1b1* in the oviductal secretory cells (Fig. 7B).

E_2 induced colocalization between ESR1 and $G\alpha_i$ in the oviductal secretory cells

In this study, we examined whether E_2 is able to induce colocalization between ESR1 and $G\alpha_i$ in the oviductal secretory cells. Primary cultures from rat oviductal secretory cells were treated with ethanol or E_2 M and 3 h later the expression and colocalization of ESR1 and $G\alpha_i$ were assessed by confocal microscopy. This experiment was replicated five times.

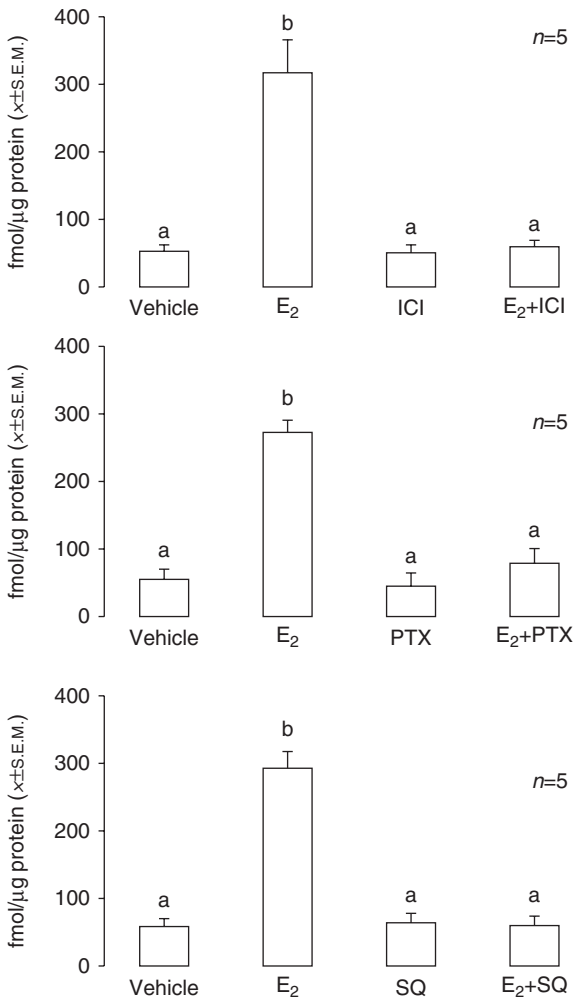

Figure 6 Effect of ICI 182 780, PTX, or SQ 22536 on the E_2-induced cAMP increase in the oviductal secretory cells. Primary cultures of secretory cells from rat oviducts were treated with E_2 10^{-9} M alone or with the estrogen receptor antagonist ICI 182 780 25 µg/µl, the $G\alpha_i$ protein inhibitor PTX 25 µg/µl, or the adenylyl cyclase inhibitor SQ 22536 7.5 µg/µl and 3 h later the cAMP level was determined by an enzyme immunoassay system. Note that all three inhibitors blocked the effect of E_2 on the level of cAMP. This experiment consisted of five replicates. a≠b, $P<0.05$.

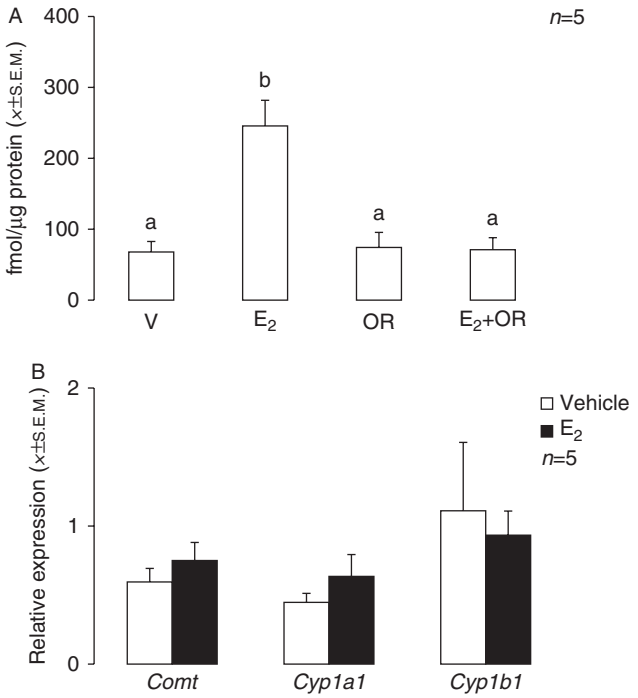

Figure 7 Effect of OR 486 on the E_2-induced cAMP increase and expression of *Comt*, *Cyp1a1*, and *Cyp1b1* transcripts in the oviductal secretory cells. (A) Primary cultures of secretory cells from rat oviducts were treated with E_2 10^{-9} M alone or with the cathecol-*O*-methyltransferase activity inhibitor OR 486 25 µg/µl and 3 h later the cAMP level was determined by an enzyme immunoassay system. Note that OR 486 blocked the effect of E_2 on the cAMP level. This experiment consisted of five replicates. a≠b, $P<0.05$. (B) Primary cultures of secretory cells from rat oviducts were treated with E_2 10^{-9} M and 3 h later the relative expression of the mRNA for *Comt*, *Cyp1a1*, and *Cyp1b1* was determined by real-time PCR. The values were normalized to *Gapdh*. Note that E_2 did not change the level of the transcripts.

In the control group, ESR1 distribution was found in the nuclear and extranuclear sites as previously reported for the rat oviductal epithelial cells (Orihuela *et al.* 2009), but colocalization was not observed between ESR1 and $G\alpha_i$ proteins. However, E_2 administration induced colocalization between ESR1 and $G\alpha_i$ in the regions outside the nucleus of the oviductal secretory cells (Fig. 8).

Discussion

The E_2 nongenomic pathway that accelerates egg transport involves sequential activation of the signaling cascades of cAMP–PKA–PLC–IP3 in the rat oviduct (reviewed in Orihuela *et al.* (2013)). However, the role of the different cell phenotypes of the rat oviduct on this E_2 nongenomic pathway is still unknown. Herein, we show for the first time the separate contributions of the secretory and smooth muscle cells on the dynamic process that modulates cAMP and IP3 levels in response to an E_2 pulse. In the secretory cells, E_2 increased cAMP production between 3 and 6 h, although the response of IP3 was not affected. In the smooth muscle cells, E_2 transiently decreased cAMP level at 3 h while IP3 level was increased at 6 h. Thus, E_2 differentially regulates cAMP and IP3 production in the secretory and smooth muscle cells of the rat oviduct. These differences may be attributed to the differential expression of the ER subtypes that exist in the secretory and smooth muscle cells of the rat oviduct (Mowa & Iwanaga 2000, Orihuela *et al.* 2009) or to changes in the different pools of ER that initiate E_2 nongenomic actions between these two cellular phenotypes (Orihuela *et al.* 2009).

Further investigation to disclose the signaling pathway by which E_2 increases the level of cAMP in the secretory cells of the rat oviduct revealed that this effect of E_2 was by a nongenomic mechanism because suppression of mRNA protein synthesis by ActD did not prevent the effect of E_2 on the cAMP level. Blockade of ER by ICI 182 780 and inhibition of AC by SQ 22536 suppressed the E_2-induced cAMP increase in the secretory cells of the rat oviduct indicating that the E_2 nongenomic pathway that increases cAMP requires binding of the hormone to its classical receptor and activation of AC. Several works have implicated to the E_2 nongenomic actions with the intracellular cAMP-signaling cascade. E_2 activates AC in vascular smooth muscle, breast cancer, and uterine cells by a nongenomic mechanism (Aronica *et al.* 1994, Farhat *et al.* 1996), while acute stimulation of Ca^{2+} uptake induced by E_2 is accompanied by increased cAMP content in rat duodenal cells and preosteoclastic cells (Fiorelli *et al.* 1996, Picotto *et al.* 1996). Our findings provide the first evidence of a nongenomic action of E_2 associated to a cAMP increase in the secretory cells of the mammalian oviduct. Probably, this E_2 nongenomic action could be associated to the regulation of the secretory activity necessary to

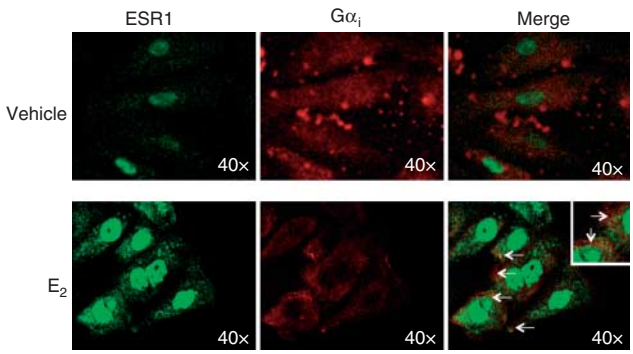

Figure 8 Estradiol induces colocalization between ESR1 and $G\alpha_i$ in the oviductal secretory cells. Representative photomicrographs obtained from primary cultures of oviductal secretory cells treated with E_2 10^{-9} M or vehicle and 3 h later the colocalization between ESR1 (green) and $G\alpha_i$ (red) were determined by confocal microscopy. The merged image display colocalization of ESR1 and $G\alpha_i$ as an orange signal (arrows) only in the group treated with E_2. Magnification is shown in the inset.

accelerate egg transport in the rat oviduct. Alternatively, regulation of the tubal fluid formation by an increase in the level of cAMP may be useful in providing an adequate environment for some reproductive events as sperm migration, oocyte fertilization, or preimplantation embryo development. According with this assumption, previous works have reported that estrogens regulate the formation of tubal fluid secreted by the oviductal epithelial cells (Leese *et al*. 2001) and that cAMP increases fluid secretion into the oviductal lumen of several species (Leung *et al*. 1995, Chen *et al*. 2010, Liao *et al*. 2013).

There is increasing evidence that some biological effects of E_2 are in part mediated by its metabolites 2- and 4-methoxyestradiol (Mueck & Seeger 2010, Parada-Bustamante *et al*. 2013, Perez-Sepulveda *et al*. 2013), indicating an important role of methoxyestrogens in the signaling cascades of E_2 on its target organs. Our results showing that suppression of the COMT activity blocked the E_2-stimulated cAMP accumulation in the secretory cells of the rat oviduct suggest that this nongenomic action of E_2 requires previous conversion of E_2 to methoxyestrogens. Furthermore, the enzymes necessary to metabolize estrogens are present in the oviductal secretory cells, although their expression was not regulated by E_2. In order to corroborate the importance of methoxyestrogens in the nongenomic action of E_2 that increases cAMP in the oviductal secretory cells, it is necessary to treat these cells with methoxyestrogens and evaluate their effects on the level of cAMP; however, this was not explored in this work.

The role of G proteins in the nongenomic actions of E_2 has been documented in a variety of cell types (Levin 1999, Fu & Simoncini 2008). In this context, we have found that the ADP-ribosylating agent PTX blocked the effect of E_2 on the cAMP production in the secretory cells, indicating a requirement for heterotrimeric $G_{i/o}$-type proteins in this nongenomic action of E_2. This is supported by the fact that E_2 also induced colocalization between ESR1 and $G\alpha_i$ in non-nuclear sites of these cells, suggesting coupling of ESR1 with $G\alpha_i$ protein as a requisite for the E_2-induced cAMP increase. Although all major subclass of G proteins are expressed in many cell types, it appears that $G\alpha_i$ subclass is in a great mode, the one most often linked with the E_2 nongenomic actions involving the participation of a presumptive ESR1 localized in extranuclear sites (Wyckoff *et al*. 2001, Kumar *et al*. 2007, Lin *et al*. 2011, Watson *et al*. 2012). To our knowledge, this is the first report showing association between ESR1 and $G\alpha_i$ in extranuclear sites of the epithelium cells that may mediate an E_2 nongenomic action in the mammalian oviduct. On the other hand, we cannot assure whether this ESR1–$G\alpha_i$ colocalization occurs in the plasma membrane or is a consequence of diminution of the ESR1 or $G\alpha_i$ expression in the secretory cells, because subcellular fractionation or immunoprecipitation experiments were not done in this work. Further studies are necessary to disclose the molecular and cellular mechanisms that explain how the activation of the $G\alpha_i$ protein participates in the E_2 nongenomic pathway of the rat oviduct.

E_2 regulates the expression of insulin-like growth factor 1 (IGF1), IGF1-binding proteins, and IGF1 receptors in neurons of the CNS and in reproductive tissues (Wimalasena *et al*. 1993, Sahlin *et al*. 1994, Azcoitia *et al*. 1999). Furthermore, IGF1 stimulates the accumulation of cAMP in mouse astrocytes and in the preoptic area and hypothalamus of the rat. Moreover, IGF1 is able to induce activation of ER via an increase in intracellular cAMP level in the rat uterine cells (Aronica & Katzenellenbogen 1993, Bartella *et al*. 2012). Probably, the E_2-induced cAMP increase observed in the secretory cells of the rat oviduct involves functional cross talk between E_2/ER and IGF1 signaling pathways, but this remains undetermined.

In summary, we have found that E_2 differentially regulates cAMP and IP3 production in the secretory and smooth muscle cells of the rat oviduct. In the secretory cells, E_2 increases cAMP production by a nongenomic action that requires COMT and ER activation, coupling between ESR1 and $G\alpha_i$, and stimulation of AC. These findings provide new evidence for understanding the contribution of the different cellular phenotypes present in the rat oviduct on the nongenomic regulation of the egg transport exerted by E_2.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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