REPRODUCTION
RESEARCH

# Estradiol increases $IP_3$ by a nongenomic mechanism in the smooth muscle cells from the rat oviduct

Patricia Reuquén[1,2], María L Oróstica[1,2], Israel Rojas[1,2], Patricia Díaz[1,2], Alexis Parada-Bustamante[3] and Pedro A Orihuela[1,2]

[1]*Laboratorio de Inmunología de la Reproducción, Facultad de Química y Biología, Universidad de Santiago de Chile,* [2]*Centro para el Desarrollo en Nanociencia y Nanotecnología-CEDENNA and* [3]*Instituto de Investigaciones Materno-Infantil, Universidad de Chile, Alameda 3363, Casilla 40, Correo 33 Santiago, Chile*

*Correspondence should be addressed to P A Orihuela; Email: pedro.orihuela@usach.cl*

## Abstract

**Estradiol ($E_2$) accelerates egg transport by a nongenomic action, requiring activation of estrogen receptor (ER) and successive cAMP and $IP_3$ production in the rat oviduct. Furthermore, $E_2$ increases $IP_3$ production in primary cultures of oviductal smooth muscle cells. As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by $E_2$ in the oviduct, herein we determined the mechanism by which $E_2$ increases $IP_3$ in these cells. Inhibition of protein synthesis by Actinomycin D did not affect the $E_2$-induced $IP_3$ increase, although this was blocked by the ER antagonist ICI182780 and the inhibitor of phospholipase C (PLC) ET-18-$OCH_3$. Immunoelectron microscopy for ESR1 or ESR2 showed that these receptors were associated with the plasma membrane, indicating compatible localization with $E_2$ nongenomic actions in the smooth muscle cells. Furthermore, ESR1 but not ESR2 agonist mimicked the effect of $E_2$ on the $IP_3$ level. Finally, $E_2$ stimulated the activity of a protein associated with the contractile tone, calcium/calmodulin-dependent protein kinase II (CaMKII), in the smooth muscle cells. We conclude that $E_2$ increases $IP_3$ by a nongenomic action operated by ESR1 and that involves the activation of PLC in the smooth muscle cells of the rat oviduct. This $E_2$ effect is associated with CaMKII activation in the smooth muscle cells, suggesting that $IP_3$ and CaMKII are involved in the contractile activity necessary to accelerate oviductal egg transport.**

*Reproduction* (2015) **150** 331–341

## Introduction

The canonical pathway by which estradiol ($E_2$) affects its target cells comprises binding to estrogen receptors (ER) and modification of gene expression and protein synthesis (Nilsson *et al*. 2001). However, some $E_2$ effects cannot be blocked by inhibitors of transcription or translation, or 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 *et al*. 2003, Lössel & Wheling 2003). $E_2$ nongenomic actions often involve the activation of G protein-$\alpha$ inhibitory ($G\alpha_i$), stimulation of intracellular signal transduction pathways, including the generation of second messengers such as cAMP and $IP_3$, 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).

In the rat, a single injection of $E_2$ on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 h to <24 h (Ortíz *et al*. 1979). We have previously demonstrated that RNA and protein synthesis inhibitors did not block $E_2$-induced oviductal egg transport acceleration 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 through the activation of catechol-*O*-methyltransferase (COMT) (Parada-Bustamante *et al*. 2007, 2010), ER and adenylyl cyclase (AC) (Orihuela *et al*. 2003), and sequential production of cAMP and $IP_3$ (Orihuela *et al*. 2003, 2006, 2013).

The rat oviduct is mainly composed of an intrinsic layer of smooth muscle fibre, and an innermost highly folded mucosa formed by epithelial and stromal cells, the endosalpinx (reviewed in Croxatto (2002)). Transport of oocytes along the oviduct depends on the interaction between the secretory activity of the epithelial cells and the contractile activity of the smooth muscle cells (Moore & Croxatto 1988*a*,*b*, Ríos *et al*. 2007). The regulation of muscular motility is influenced by $E_2$ and requires activity of adrenergic nerves (Helm *et al*. 1982), nitric oxide (Perez Martinez *et al*. 2000), endothelin (Parada-Bustamante *et al*. 2012), oxytocin (Jankovic *et al*. 2001) and prostaglandins (Wijayagunawardane *et al*. 2003). These factors activate intracellular


ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-15-0137
Online version via www.reproduction-online.org

signalling mainly associated with $Ca^{2+}$, cAMP or $IP_3$ (Jankovic *et al*. 2001, Barrera *et al*. 2004, Mohan *et al*. 2012). In this context, we have recently shown that, in the epithelial cells of the rat oviduct, $E_2$ increased cAMP production between 3 and 6 h, although $IP_3$ levels were not affected. Moreover, $E_2$ increased cAMP in the oviductal epithelial cells by a nongenomic mechanism that requires coupling between ESR1 and $G\alpha_i$ and stimulation of AC (Oróstica *et al*. 2014). Previous research has also shown that $E_2$ increased $IP_3$ levels in primary cultures of smooth muscle cells from the rat oviduct (Oróstica *et al*. 2014).

As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by $E_2$ in the oviduct (Croxatto 2002), this work determined the mechanism by which $E_2$ increases $IP_3$ in primary cultures of rat oviductal smooth muscle cells. Thus, we examined the effect of $E_2$ on the $IP_3$ levels in the smooth muscle cells under conditions in which protein synthesis, ER, $G\alpha_i$ or PLC activity were blocked by selective inhibitors. The subcellular localization of ESR1 and ESR2 as well as the effect of selective agonists for ESR1 or ESR2 on the $IP_3$ level was evaluated in the smooth muscle cells. Furthermore, expression of $G\alpha_i$ in the oviductal smooth muscle cells was also determined. Finally, the effect of $E_2$ on the activity of the enzyme associated to muscle contraction calcium–calmodulin protein kinase II (CaMKII) was determined in the primary cultures of smooth muscle cells.

## Materials and methods

### Animals

Locally bred Sprague-Dawley rats weighing 200–260 g were used. 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 estrous stage. The phases of the estrous cycle were determined by daily vaginal smears (Turner 1961) and all females were used after showing 2 consecutive 4-day cycles. The Ethical Committees of the Universidad de Santiago de Chile and the National Fund of Science (CONICYT-FONDECYT 1110662) approved the protocols for the care and manipulation of the animals.

### Culture of primary smooth muscle cells from rat oviducts

For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hanks's solution (Sigma Chemical) at pH 7.4. The whole oviduct was cut into small (4–8 $mm^2$) pieces in Hanks's 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 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, Franklin Lakes, NJ, USA) in DMEM/High Modified medium with 4.0 mM L-glutamine and 4.5 g/l glucose free of Phenol Red (Cat. No. SH30284.02, HyClone, Thermo Scientific, Waltham, MA, USA) supplemented with 10% (v/v) foetal bovine serum (Cat. No. SH30396.03, HyClone), 1 mM sodium pyruvate and 100 UI/ml penicillin and 100 µg/ml streptomycin. Smooth muscle cells were incubated at 37 °C in an atmosphere of 5% (v/v) $CO_2$ for at least 7 days to reach 75–80% confluence and their purity verified by immunofluorescence staining for cytokeratin (marker of epithelium cells), vimentin (marker of fibroblasts) or α-actin (marker of smooth muscle cells) antibodies.

### Treatments

Primary cultures of smooth muscle cells were changed to medium without serum for 15 h before each treatment. Then, they were treated with $10^{-9}$ M $E_2$ (Sigma) or 0.01% ethanol as vehicle. Other primary cultures of smooth muscle cells were also incubated with the protein synthesis inhibitor 1 µg/µl Actinomycin D (ActD, Goldberg *et al*. 1962, Sigma), the ER antagonist 25 µg/µl ICI 182780 (Gagliardi & Collins 1993, Tocris Bioscience, Bristol, UK), the PLC inhibitor 1 µM ET-18-$OCH_3$ (Powis *et al*. 1992, Calbiochem, La Jolla, CA, USA) or the $G\alpha_i$ protein inhibitor pertussis toxin 1 µg/ml PTX (Lee *et al*. 2002, Sigma) as appropriate to each experiment. Furthermore, the ESR1 agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole ($10^{-7}$–$10^{-5}$ M PPT, Sigma) or ESR2 agonist Diarylpropionitrile ($10^{-7}$–$10^{-5}$ M DPN, Sigma) were added to the primary smooth muscle cells. The concentrations of PPT and DPN used in this work were based considering that both drugs have lower affinity for ESR1 and ESR2 than $E_2$ (Harris *et al*. 2002, Frasor *et al*. 2003, Harrington *et al*. 2003). DMSO at 0.01% was used as a vehicle for the inhibitors and agonists because it is more efficient than ethanol at dissolveing nonpolar or semi-polar drugs.

### Extraction and measurement of $IP_3$

Primary smooth muscle cell cultures were sonicated in 100 µl of ice-cold 1 M trichloroacetic acid (TCA) and an aliquot was taken to measure protein concentration by the Bradford assay using BSA dissolved in 1 M TCA as standard (Bio-Rad). The remaining homogenate was then 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-trifluoroethane (TCTFE, Sigma)-Trioctylamine (Sigma), 3:1 (v/v). Levels of $IP_3$ were determined using $IP_3$ [$^3$H] radioreceptor assay Kit, Cat. No NEK064 (NEN Life Science Products, Boston, MA, USA). This kit is based on competition between non-radioactive $IP_3$ and a fixed quantity of [$^3$H]-$IP_3$, for a limited number of calf cerebellum $IP_3$ receptor binding sites. This allows the construction of a standard curve and the measurement of $IP_3$ levels in unknown samples.

### Western blot

Polyclonal antibodies that recognize the phosphorylated state of CaMKII on Thr286 (anti-phospho-CaMKII, Cell Signaling Technology, Beverly, MA, USA) or total CaMKII (anti-CaMKII,

Abcam, Cambridge, UK) were used to assess activation of CaMKII. Smooth muscle cells were processed by duplicate to determine the activity of the CaMKII protein. Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with a protease inhibitor cocktail (Roche Diagnostics). The lysate was centrifuged at 4 °C for 10 min at 10 000 ***g*** and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad). After boiling for 5 min, proteins (20 μg) were separated on 10% SDS–PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked 3 h in TTBS (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) that contained 5% nonfat dry milk and were incubated overnight with 0.4 μg/ml rabbit anti-phospho-CaMKII (Cell Signaling Technology). The immunoreactive band was visualized by incubation for 1 h with 0.04 μg/ml goat anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) conjugated to HRP, followed by the Enhanced Western Lighting Chemiluminescence reaction (PerkinElmer Life Sciences, Boston, MA, USA). Blots were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl, pH 6.7 at 60 °C for 30 min, and reprobed with 0.2 μg/ml rabbit anti-CaMKII antibody and developed in a similar manner to ensure even loading. All blots were then digitalized and the relative level of phospho-CaMKII was normalized against total CaMKII. Oviductal samples without anti-phospho-CaMKII or anti-CaMKII antibody were included as negative controls.

### *Immunoelectron microscopy*

A post-embedding immunogold-labelling method that preserves cellular integrity and maintains ER immunogenicity was used (Kessels *et al.* 1998, Qualmann *et al.* 2000, Orihuela *et al.* 2009). Primary smooth muscle cell cultures were fixed in 4% freshly depolymerised paraformaldehyde, 0.5% glutaraldehyde in 1 M phosphate buffer pH 7.4 containing 0.1 M saccharose, 1% DMSO and 1% $CaCl_2$ for 2–4 h at room temperature. The fixed samples were washed three times with phosphate buffer, dehydrated in a graded ethanol series and infiltrated with LR Gold (Plano, München, Germany). Subsequently, the samples were transferred to gelatin capsules filled with 0.8% (w/v) benzoyl peroxide in LR Gold and kept for polymerization at a pressure of 500 mmHg. The blocks were cured for 1–2 days at room temperature before sectioning with a Sorvall-2000 ultramicrotome using a diamond knife. The sections (50–80 nm) were mounted on formvar-coated nickel grids and incubated on droplets of 0.1 M glycine in PBS pH 7.6, and subsequently blocked with 1% bovine fetal serum for 2 h at room temperature. The grids were then incubated for 2 h with 4.0 μg/ml rabbit anti-ESR1 (MC-20, Santa Cruz Biotechnology) or 4.0 μg/ml anti-ESR2 (clone 68-4, Chemicon International). After washing with PBS, the preparations were incubated for 1 h with 0.3 μg/ml goat anti-rabbit immunoglobulin conjugated to 10 nm gold particles (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Sections were washed and contrasted with Reynolds stain (Reynolds 1963). All sections were examined using a Phillips-TECNAI 12 BIOTWIN EM Microscope (FEI Company, Hillsboro, OR, USA) at 80 kV. As negative control for ESR1 and ESR2, the primary antibody was replaced by preimmune serum. At least ten areas of 63 $\mu m^2$ from different smooth muscle cells from the primary cultures were photographed and the photomicrographs were digitalized in an iBook computer (Apple Computer, Cupertino, CA, USA), and gold particles present only in the cells were counted using the image analysis Software Adobe Photoshop 7.0 (Adobe Systems) by an observer blinded to the different groups. The results of the immunolabelling are presented as the quotient of the number of gold particles present divided by the area and cell number analysed in three different cultures (Orihuela *et al.* 2009).

### *Immunofluorescence*

Smooth muscle cells were fixed in cold 4% paraformaldehyde in PBS pH 7.4–7.6 for 2 h, transferred to 10% (w/v) saccharose in PBS for 60 min at 4 °C and 30% (w/v) saccharose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS–BSA for 120 min, and incubated with 0.8 μg/ml mouse anti-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2.5 μg/ml mouse anti-vimentin (Santa Cruz Biotechnology), 0.4 μg/ml mouse anti-α-actin (Santa Cruz Biotechnology) or 5.0 μg/ml mouse anti-$G\alpha_i$ (Santa Cruz Biotechnology) antibodies. After washing with PBS, the preparations were incubated for 2 h with 0.5 μg/ml Alexa fluor 588-conjugated goat anti-mouse IgG (Invitrogen). Sections were washed and counterstained with 1 μg/ml of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) washing again and then mounted in Fluoromount G. As negative controls, the primary antibody was replaced by preimmune serum. As positive control for $G\alpha_i$ we used samples of whole oviducts from rats on day 1 of the estrous cycle (Oróstica *et al.* 2013, 2014). All sections were visualized with an Optiphot Epifluoresence Microscope (Olympus).

### *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 reverse transcription 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) was used to quantify the relative gene expression of the $E_2$-target genes *c-fos* (Nilsson *et al.* 2001) in the oviductal smooth muscle 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 *c-fos* were 5′-CCG AGA TTG CCA ATC TAC TG 3′ (sense) and 5′-AGA AGG AAC CAG ACA GGT CC 3′ (antisense) and for *Gapdh* were 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 performed in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 5 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 (60° up to 95 °C). The expression of *c-fos* was determined using the equation:

$Y = 2^{-\Delta Cp}$ (16) 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 *c-fos* 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 and Schmittgen 2001).

### *Statistical analysis*

Data for $IP_3$ and CaMKII assays 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). Statistical analysis was performed using a GraphPad Prism 5.0 Software program. All data are presented as mean ± S.E.M. These data followed a non-normal distribution (Kolmogorov–Smirnov test) and significant differences between groups were determined through the use of variance analysis by Friedman's test with subsequent *post-hoc* Wilcoxon signed-rank test. Significance was accepted at $P<0.05$. On the other, the quantitative analysis of the ESR1 or ESR2 distribution was subjected to Kruskal-Wallis test, followed by Mann-Whitney's *U* tests for pairwise comparisons when overall significance was detected. Significance was accepted at $P<0.05$.

## Results

### *$E_2$ increased $IP_3$ production by a nongenomic action in the smooth muscle cells from the rat oviduct*

Primary cultures of smooth cells from rat oviducts, with a purity of 90–95% (Fig. 1), were divided into the following treatment groups: i) ethanol + DMSO, ii) $E_2$ + DMSO, iii) ethanol + ActD and iv) $E_2$ + ActD. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section.

Figure 2 shows that in the vehicle group, $IP_3$ production ranged from 114.1 ± 30.3 to 123.2 ± 26.7 fmol/μg of protein while in the $E_2$-treated group it was increased at 6 h (523.2 ± 66.7 fmol/μg of protein) but not at 1, 3 or 9 h. Administration of ActD alone or concomitant with $E_2$ did not affect the time-course of $IP_3$ production in the control or in the $E_2$-treated group.

### *$E_2$ increased $IP_3$ production through ER and PLC activation in the oviductal smooth muscle cells*

In each experiment, primary cultures of smooth muscle 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 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section.

Figure 3 shows that in the control groups, the $IP_3$ level ranged from 151.6 ± 26.3 to 193.5 ± 37.3 fmol/μg of protein while in the $E_2$-treated groups it ranged from 488.3 ± 59.7 to 599.4 ± 71.3 fmol/μg of protein. Administration of ICI 182780 or ET-18-$OCH_3$ alone did not affect the basal $IP_3$ production although blocked the $E_2$-simulated $IP_3$ increase at 6 h.

### *ESR1 and ESR2 are localized in association with the plasma membrane of the oviductal smooth muscle cells*

Primary cultures of smooth muscle cells from rat oviducts with no treatment were processed by immunoelectron microscopy using specific antibodies for ESR1 and ESR2.

Figure 4A shows that immunoreactivity for ESR1 and ESR2 was found associated to the plasma membrane, cytoplasm and nucleus in the oviductal smooth muscle cells. Furthermore, Fig. 4B shows that the quantitative analysis of the ESR1 or ESR2 distribution was a higher number of ESR1 and ESR2-reacting gold particles in the nucleus than in the plasma membrane or cytoplasm of the smooth muscle cells.

### *Activation of ESR1 but not ESR2 mimic the effect of $E_2$ on the $IP_3$ production in the oviductal smooth muscle cells*

Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, PPT ($10^{-7}$–$10^{-5}$ M) or DPN ($10^{-7}$–$10^{-5}$ M) during 0, 1, 3, 6 or 9 h and processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section. Other smooth muscle cells cultures were also treated with

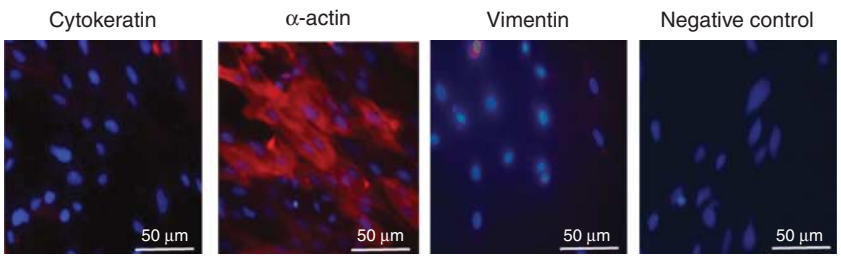


**Figure 1** Expression of α-actin in primary cultures of smooth muscle cells from rat oviducts. Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts were processed by immunofluorescence microscopy to detect expression of cytokeratin, vimentin or α-actin. Note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

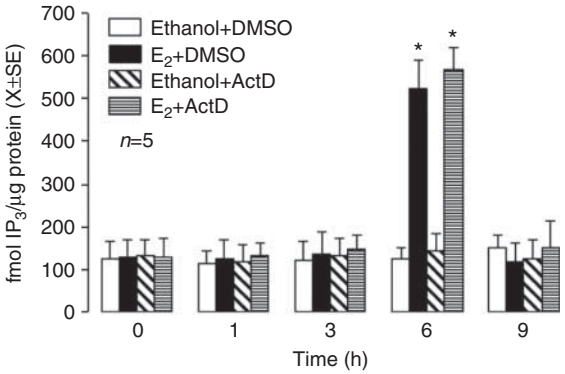


**Figure 2** Effect of Actinomycin D (ActD) on the $E_2$-induced $IP_3$ increase in the oviductal smooth muscle cells. Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) $E_2$ + DMSO, iii) ethanol + ActD and iv) $E_2$ + ActD. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section. Note that ActD did not block the effect of $E_2$ on the $IP_3$ level. This experiment consisted of five replicates. *$P<0.05$ with respect to each treatment group.

DMSO or DPN $10^{-7}$ M and 6 h later processed by Real-Time PCR to determine the mRNA level of *c-fos*.

Figure 5 shows that in the control group, the $IP_3$ production ranged from 120.3±44.1 to 148.5±50.6 fmol/µg of protein while treatment with PPT increased $IP_3$ level in a dose-dependent manner at 6 h without any effect at 0, 1, 3 or 9 h. On the other hand, administration of DPN had no effect on the $IP_3$ level at any time or concentration studied (Fig. 5), although it increased the mRNA level of *c-fos* (control: 51.4±11.3 vs DPN: 210.7±36.2 relative expression, $n=5$).

### *$G\alpha_I$ protein is not required for the $IP_3$ production increase induced by $E_2$ in the oviductal smooth muscle cells*

Primary cultures of smooth cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) $E_2$ + DMSO. iii) ethanol + PTX and iv) $E_2$ + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section.

Figure 6A shows that in the control group, $IP_3$ production ranged from 124.9±38.1 to 133.5±36.9 fmol/µg of protein while in the $E_2$-treated group it was increased at 6 h (601.4±98.5 fmol/µg of protein) but not at 1, 3 or 9 h. Administration of PTX alone or concomitant with $E_2$ did not affect the time-course of $IP_3$ production in the control or in the $E_2$-treated group.

### *$G\alpha_I$ protein is not expressed in the smooth muscle cells from the rat oviduct*

Primary cultures from smooth muscle cells from rat oviducts were processed by immunofluorescence in order to assess the immunoreactivity of $G\alpha_I$ in these cells. In addition, we used sections of whole oviducts from rats on day 1 of the oestrous cycle to corroborate presence of $G\alpha_i$ in the oviductal tissues. This experiment was replicated five times.

Figure 6B shows that immunoreactivity of $G\alpha_I$ was not found either in the primary cultures of the oviductal smooth muscle cells or in the myosalpinx layer of the whole oviduct. However, $G\alpha_I$ was expressed in the endosalpinx of the rat oviduct as previously reported by Oróstica *et al.* (2014).

### *$E_2$ induced activation of CaMKII in the oviductal smooth muscle cells*

Primary cultures from rat oviductal secretory cells were treated with ethanol or $E_2$ $10^{-9}$ M and 6.5 h later the level of phosphorylated CaMKII (p-CaMKII) was assessed by immunoblot. As $E_2$ increases the $IP_3$ level at 6 h after

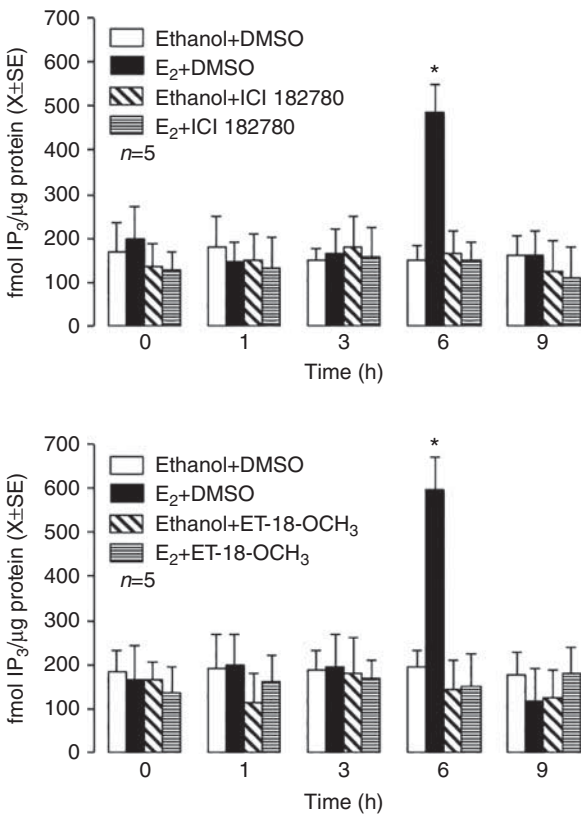


**Figure 3** Effect of ICI 182780 or ET-18-$OCH_3$ on the $E_2$-induced $IP_3$ increase in the oviductal smooth muscle cells. In each experiment, primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) $E_2$ + DMSO, iii) ethanol + inhibitor and iv) $E_2$ + inhibitor. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section. Note that ICI 182780 and ET-18-$OCH_3$ blocked the effect of $E_2$ on the level of $IP_3$ at 6 h. This experiment consisted of five replicates, *$P<0.05$ with respect to each treatment group.

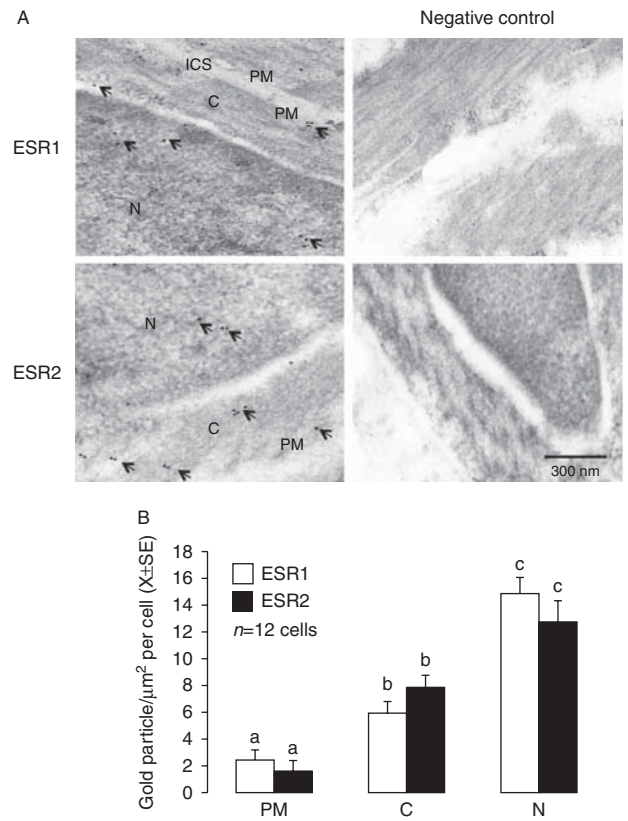


**Figure 4** Subcellular localization of ESR1 and ESR2 in the primary cultures of smooth muscle cells from rat oviducts. (A) Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts processed by immunoelectron microscopy with gold labeled-antibodies for ESR1 or ESR2. Arrows show gold particle labeling localized to the plasma membrane (PM), cytoplasm (C) and nucleus (N). Negative controls of the immunoreactivity were incubated with preimmune serum. ICS = intercellular space. (B) Density of gold particles (X ± SE) observed for ESR1 and ESR2 in the plasma membrane (PM), cytoplasm (C) and nucleus (N). Note that density of ESR1 and ESR2 in the PM were lower than in the C and N. $a \neq b \neq c$, $P<0.05$. Replicates of this experiment consisted of 12 cells from three different cell cultures (four individuals cells/well plate).

treatment, we consider that 6.5 h is a reasonable time to evaluate activation of CaMKII downstream of the $IP_3$ increase.

Figure 7 shows that $E_2$ increased the level of p-CaMKII in comparison with the vehicle group.

## Discussion

The contribution of the different cell phenotypes of the rat oviduct on the $E_2$ nongenomic pathway associated with the cAMP-$IP_3$ signalling and involved in the accelerated egg transport is recently being disclosed (reviewed in Orihuela *et al.* (2013)). Here we show that $E_2$ increased $IP_3$ levels in the oviductal smooth muscle cells by a nongenomic mechanism because suppression of mRNA and protein synthesis by ActD did not prevent the effect of $E_2$ on the $IP_3$ level. Moreover, the $E_2$ nongenomic pathway that increases $IP_3$ requires activation of ER and PLC since blockade of ER by ICI 182780 and PLC by ET-18-$OCH_3$ reverted the $E_2$-induced $IP_3$ increase in the oviductal smooth muscle cells. Previous works have shown that some $E_2$ nongenomic pathways are associated with changes in the turnover of inositol lipids that generates $IP_3$ from the hydrolysis of phosphatidylinositol 4,5-biphosphate in several cell systems (Kisielewska *et al.* 1996, 1997, Razandi *et al.* 1999, Ariazi *et al.* 2010). Our findings show for the first time that a nongenomic action of $E_2$ associated with PLC-$IP_3$ signalling is also present in the smooth muscle cells of the mammalian oviduct. The effect of $E_2$ on the $IP_3$ level occurred from 6 h and declined at 9 h, indicating a transient action on the PLC-$IP_3$ signalling in the smooth muscle cells. A rapid turnover of $IP_3$, inactivation of PLC or down-regulation of ER could explain the lack

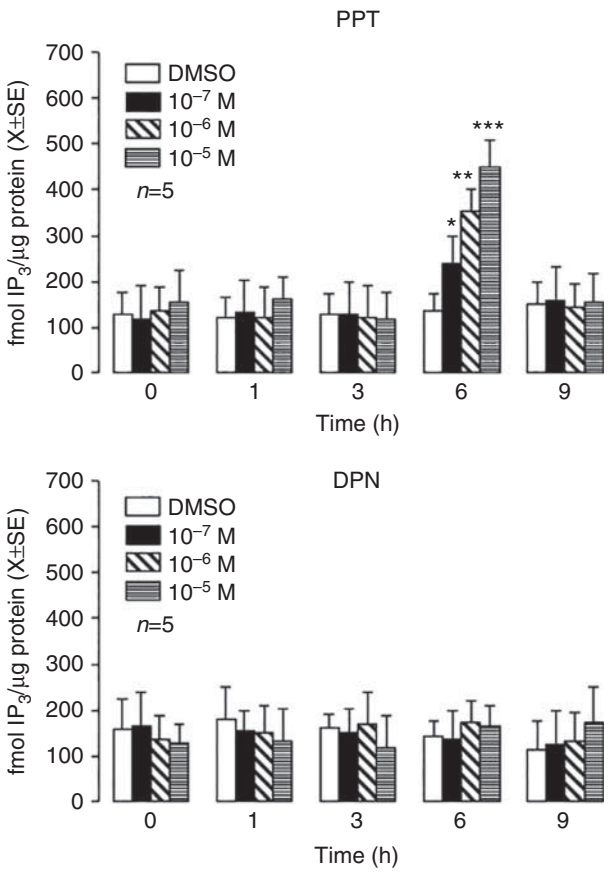


**Figure 5** Effect of selective agonists for ESR1 (PPT) or ESR2 (DPN) on the $IP_3$ level in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, $10^{-7}$–$10^{-5}$ M PPT or DPN during 0, 1, 3, 6 or 9 h and processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section. Note that PPT increased the $IP_3$ level in a concentration-dependent manner at 6 h after treatment while DPN had no any effect. This experiment consisted of five replicates. $^*P<0.05$, $^{**}P<0.02$ and $^{***}P<0.01$ with respect to each treatment group.

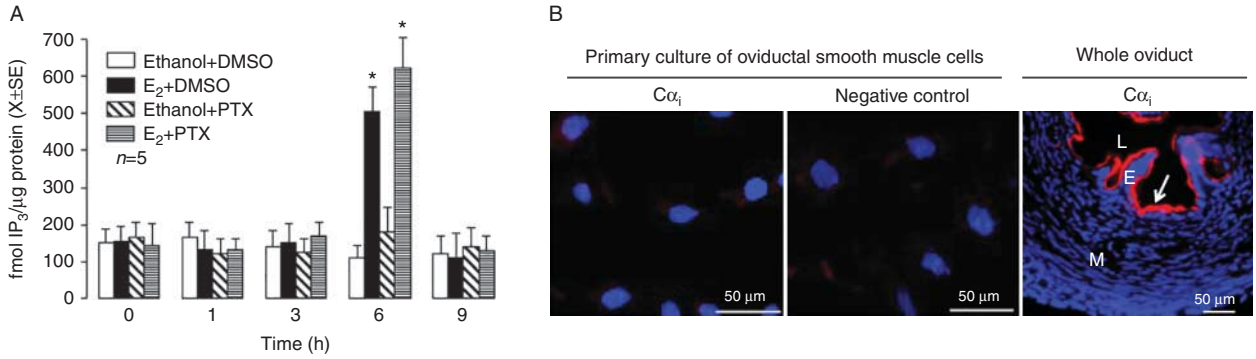


**Figure 6** Effect of PTX on $E_2$-induced $IP_3$ increase and expression of $G\alpha_i$ in the oviductal smooth muscle cells. (A) Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) $E_2$ + DMSO, iii) ethanol + PTX and iv) $E_2$ + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of $IP_3$ as described in the 'Materials and methods' section. Note that PTX did not block the effect of $E_2$ on the $IP_3$ level. This experiment consisted of five replicates. *$P<0.05$ with respect to each treatment group. (B) Representative photomicrographs obtained from primary cultures of oviductal smooth muscle cells or whole oviduct processed by immunofluorescence to detect expression of $G\alpha_i$ protein. Note that Gai (red) is only expressed in the endosalpinx (mainly composed of epithelium cells) layer of the whole oviduct. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum. L, lumen; E, endosalpinx; M, myosalpinx.

of effect $E_2$ on the $IP_3$ level at 9 h; however, this remains to be determined.

In contrast to other reports that showed a rapid increase of $IP_3$ by $E_2$ in rat vaginal epithelial cells and HEPG2 cells (Singh & Gupta 1997, Marino *et al*. 1998), we found that $E_2$ has a time of latency of 6 h to exert its effects on the $IP_3$ production in the smooth muscle cells from the rat oviduct. Differences in the expression of the ER isoforms or in the signalling pathways between the different cell phenotypes may explain the delayed response to $E_2$ in the smooth muscle cells. We postulate that the $E_2$ nongenomic action that increases $IP_3$ appears as a secondary response to intracellular changes localized upstream of PLC activation in the oviductal smooth muscle cells. According to this assumption, we have recently shown that $E_2$-induced $IP_3$ increase is preceded by a cAMP decrease in smooth muscle cells of the rat oviduct (Oróstica *et al*. 2014). Alternatively, $E_2$ may be first metabolized into 2-methoxyestradiol to increase $IP_3$ production in the oviductal smooth muscle cells. In this context, various biological effects of $E_2$ including regulation of egg transport in the rat oviduct or modulation of the antihypertensive and neuroprotective effects of $E_2$, requires previous conversion from $E_2$ to 2ME in its target organs (reviewed in Dubey & Jackson (2001) and Parada-Bustamante *et al*. (2015)).

Inhibition of the ER activity did not affect basal $IP_3$ production in the oviductal smooth muscle cells, indicating that other ER-independent signalling pathways are acting to state basal $IP_3$ level. In according with this idea, various signalling pathways such as Angiotensin-II, arachidonic acid, endothelin-1 and norepinephrine regulate production of $IP_3$ (reviewed in Bolton (2006)). On the other hand, ET-18-$OCH_3$ alone had no effect on the $IP_3$ production in the oviductal smooth muscle cells. Since 13 mammal PLC subtypes have actually been reported (Rhee 2001), it is probable that basal $IP_3$ production depends on an ET-18-$OCH_3$-insensitive PLC. In this context, it has been found that ET-18-$OCH_3$ is more effective at inhibiting membrane-associated PLC-β1 than PLC-γ1 localized in the cytosol of human fibroblasts (Powis *et al*. 1992).

Physiological effects of $E_2$ are mainly influenced by the differential distribution of ESR1 and ESR2 in its target organs. Our results showing localization of ESR1 and ESR2 in the cell membrane, cytoplasm and nucleus

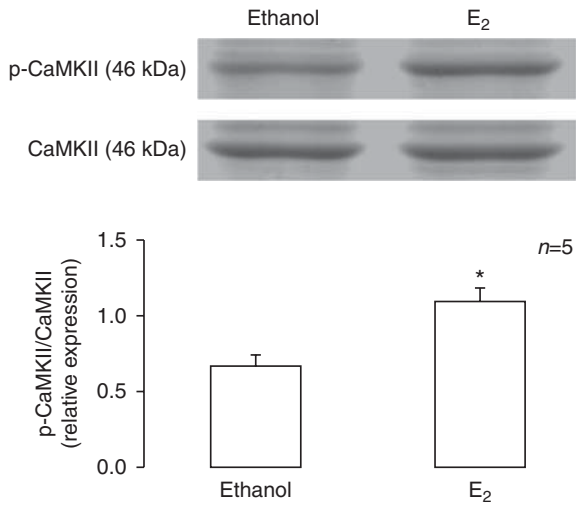


**Figure 7** Effect of $E_2$ on the CaMKII activation in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with ethanol or $E_2$ and 6.5 h later CaMKII activation was determined by western blot and densitometry using anti-phospho-CaMKII or total CaMKII antibodies. Note that $E_2$ treatment increased the relative phosphorylation of CaMKII in the smooth muscle cells. This experiment consisted of five replicates. *$P<0.05$ with respect to the control group.

indicate compatible localization of both ER subtypes with genomic and nongenomic actions of $E_2$ in the smooth muscle cells of the rat oviduct. These findings corroborate previous studies on the presence of ER in nuclear and extranuclear regions in the rat oviductal epithelium cells (Orihuela *et al*. 2009) and reinforces the concept that signalling pathways associated with $E_2$ nongenomic and genomic actions operate in the mammalian oviduct (Parada-Bustamante *et al*. 2010, 2012, Orihuela *et al*. 2013). The fact that a lower proportion of ESR1 and ESR2 was found in the plasma membrane compared with those present in the cytoplasm and nucleus of the oviductal smooth muscle cells is consistent with the notion that only a small pool of ER is responsible for the $E_2$ nongenomic actions (reviewed inWatson & Gametchu (2003) and Wehling *et al*. (2006)).

The functional relevance of ER subtypes on the $E_2$-induced $IP_3$ increase in the oviductal smooth muscle cells was investigated utilizing selective agonists for ESR1 (Harris *et al*. 2002) or ESR2 (Frasor *et al*. 2003). We found that activation of ESR1 by PPT mimicked the effect of $E_2$ on the $IP_3$ levels while activation of ESR2 by DPN had no effect. This suggests that the nongenomic pathway by which $E_2$ increases $IP_3$ in the oviductal smooth muscle cells operates through ESR1 activation. This is in keeping with previous works showing that the nongenomic actions of estrogenic compounds require activation of ESR1 (reviewed in Marino *et al*. (2006), Moenter & Chu (2012) and Watson *et al*. (2014)). On the other hand, the $E_2$ nongenomic pathways associated with activation of ESR2 in the smooth muscle cells of the rat oviduct needs to be disclosed.

It has been documented that the $G\alpha_i$ subclass is the G protein most often linked with the $E_2$ nongenomic actions coupled with a presumptive ESR1 localized in extranuclear sites (Wyckoff *et al*. 2001, Kumar *et al*. 2007, Lin *et al*. 2011, Watson *et al*. 2012). However, the ADP-ribosylating agent pertussis toxin did not block the effect of $E_2$ on the $IP_3$ production in the smooth muscle cells, suggesting that heterotrimeric $G_{i/o}$-type proteins are not required for this $E_2$ nongenomic action. This is corroborated by the fact that the $G\alpha_i$ protein was not expressed either in the primary cultures of smooth muscle cells or in the myosalpinx layer (mainly composed of smooth muscle fibers) of whole oviducts from estrous rats. $G\alpha_s$-, Gq- or $G\beta\gamma$-coupled receptors and tyrosine kinase receptors also activate PLC subtypes in a variety of cell systems (Zhu & Birnbaumer 1996, Yang *et al*. 2013). Furthermore, small Rho and Ras GTPases participate in the activation of the PLC-ε (Yang *et al*. 2013). Since differential expression of PLC subtypes and G proteins is unknown in the smooth muscle cells from the rat oviduct, further studies are necessary to establish the mechanism by which the $E_2$-ER complex activate PLC in these cells.

Previous reports have shown that the cytokine Tumour Necrosis Factor-α (TNF-α) stimulated production and release of contractile mediators as prostaglandins, endothelins and nitric oxide in the bovine oviduct (Wijayagunawardane & Miyamoto 2004, Szóstek *et al*. 2011). Moreover, TNF-α modulates the effect of $E_2$ on the oviductal egg transport and TNF-α receptors are present in the rat oviduct (Oróstica *et al*. 2013). It is probable that the $E_2$ nongenomic pathway that increases $IP_3$ could be associated with TNF-α signalling in the oviductal smooth muscle cells, but this needs to be further explored.

It is well known that binding of $IP_3$ to its specific receptors (types 1, 2 and 3) localized on the intracellular compartments (nucleus, plasma membrane or endoplasmic reticulum) participates in $Ca^{2+}$ signalling by mediating intracellular $Ca^{2+}$ release, which results in activation of proteins associated with smooth muscle contraction (Kamm & Stull 1985, Kim *et al*. 2000). In this context, CaMKII is a serine/threonine kinase that is activated by $Ca^{2+}$ and Calmodulin and it is implicated in regulation of vascular tone through phosphorylation of contractile proteins, including myosin light chain kinase (MLCK), the 20-kD myosin light chain (LC20), phospholipase-α2 and the α-subunit of $Ca^{2+}$ channel (reviewed in Kim *et al*. (2008)). Furthermore, the synthetic estrogen, estradiol benzoate, is able to stimulate CaMKII activity in mouse hippocampus by a nongenomic mechanism (Sawai *et al*. 2002). Similarly, we found that $E_2$ increased CaMKII phosphorylation in the smooth muscle cells of the rat oviduct. To our knowledge, this is the first time that an effect of $E_2$ on the activity of CaMKII in the mammalian oviduct is reported. As $E_2$ activated CaMKII presumably downstream of the $IP_3$ increase, it is probable that this enzyme could be part of the PLC-$IP_3$ signalling cascades induced by estrogens to activate nongenomic actions in smooth muscle cells.

Several works have reported that $E_2$ exerts its vasoprotective actions regulating the contractile tone of the vascular, airway and myometrium smooth muscle cells (Kisielewska *et al*. 1996, Townsend *et al*. 2010, 2012, Cairrão *et al*. 2012, Holm *et al*. 2013). These effects are mainly associated with changes in the intracellular $Ca^{2++}$ mobilization associated with cAMP and $IP_3$ signalling pathways (Kisielewska *et al*. 1996, 1997, Towsend *et al*. 2010, 2012). Since $E_2$ increases the frequency of myosalpinx contractions that accelerate oviductal egg transport in the rat (Moore & Croxatto 1988*a*,*b*), we can speculate that this $E_2$ effect involves $IP_3$ production and activation of CaMKII in the smooth muscle cells. This $E_2$ effect on the oviductal egg movement could be involved in the very early maternal–embryo interactions that occur in the uterine tissues necessary to the embryo implantation (Gomez & Muñoz 2015).

In summary, we have found that $E_2$ increases $IP_3$ by a nongenomic action operated by ESR1, and that involves activation of PLC in the smooth muscle cells of the rat oviduct. Furthermore, $E_2$ activates CaMKII presumably downstream of the $IP_3$ increase in the oviductal smooth muscle cells, suggesting that $IP_3$ and CaMKII are

involved in the contractile activity of the oviduct. These findings provide new evidence to understand the molecular mechanisms underlying the role of smooth muscle cells on the $E_2$ nongenomic action that accelerates egg transport in the rat oviduct.

## 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.

## Funding

This work received financial support from grants FONDECYT # 1110662, Proyecto BASAL FBO-07 and Proyectos Basales y Vicerrectoría de Investigación, Desarrollo e Innnovación.

## References

**Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A, Visca P & Marino M** 2005 Palmitoylation-dependent estrogen receptor α membrane localization: regulation by 17β-estradiol. *Molecular Biology of the Cell* **16** 231–237. (doi:10.1091/mbc.E04-07-0547)

**Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI *et al.*** 2010 The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Research* **70** 1184–1194. (doi:10.1158/0008-5472.CAN-09-3068)

**Barrera NP, Morales B & Villalón M** 2004 Plasma and intracellular membrane inositol 1, 4, 5-trisphosphate receptors mediate the $Ca^{2+}$ increase associated with the ATP-induced increase in ciliary beat frequency. *American Journal of Physiology. Cell Physiology* **287** C1114–C1124. (doi:10.1152/ajpcell.00343.2003)

**Bolton TB** 2006 Calcium events in smooth muscles and their interstitial cells; physiological roles of sparks. *Journal of Physiology* **570** 5–11. (doi:10.1113/jphysiol.2005.095604)

**Cairrão E, Alvarez E, Carvas JM, Santos-Silva AJ & Verde I** 2012 Nongenomic vasorelaxant effects of 17β-estradiol and progesterone in rat aorta are mediated by L-type $Ca^{2+}$ current inhibition. *Acta Pharmacologica Sinica* **33** 615–624.

**Croxatto HB** 2002 Physiology of gamete and embryo transport through the fallopian tube. *Reproductive Biomedicine Online* **4** 160–169. (doi:10.1016/S1472-6483(10)61935-9)

**Dubey RK & Jackson EK** 2001 Cardiovascular protective effects of 17β-estradiol metabolites. *Journal of Applied Physiology* **91** 1868–1883.

**Frasor J, Barnett DH, Danes JM, Hess R, Parlow AF & Katzenellenbogen BS** 2003 Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) α activity by ERβ in the uterus. *Endocrinology* **144** 3159–3166. (doi:10.1210/en.2002-0143)

**Gagliardi A & Collins C** 1993 Inhibition of angiogenesis by antiestrogens. *Cancer Research* **53** 533–535.

**Goldberg I, Rabinowitz M & Reich E** 1962 Basis of actinomycin action. I. DNA binding and inhibition of RNA polymerase synthetic reaction by actinomycin. *PNAS* **48** 2094–2101. (doi:10.1073/pnas.48.12.2094)

**Gómez E & Muñoz M** 2015 Multiple-embryo transfer for studying very early maternal-embryo interactions in cattle. *Reproduction* **150** R35–R43.

**Harrington WR, Sheng S, Barnett DH, Petz LN, Katzenellenbogen JA & Katzenellenbogen BS** 2003 Activities of estrogen receptor α- and β-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. *Molecular and Cellular Endocrinology* **206** 13–22. (doi:10.1016/S0303-7207(03)00255-7)

**Harris HA, Katzenellenbogen JA & Katzenellenbogen BS** 2002 Characterization of the biological roles of the estrogen receptors, ERα and ERβ, in estrogen target tissues *in vivo* through the use of an ERα-selective ligand. *Endocrinology* **143** 4172–4177. (doi:10.1210/en.2002-220403)

**Helm G, Owman C, Sjoberg NO & Walles B** 1982 Motor activity of the human fallopian tube *in vitro* in relation to plasma concentration of oestradiol and progesterone, and the influence of noradrenaline. *Journal of Reproduction and Fertility* **64** 233–242. (doi:10.1530/jrf.0.0640233)

**Hill BJ, Gebre S, Schlicker B, Jordan R & Necessary S** 2010 Nongenomic inhibition of coronary constriction by 17ß-estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol. *Canadian Journal of Physiology and Pharmacology* **88** 147–152. (doi:10.1139/Y09-120)

**Holm A, Hellstrand P, Olde B, Svensson D, Leeb-Lundberg LM & Nilsson BO** 2013 The G protein-coupled estrogen receptor 1 (GPER1/GPR30) agonist G-1 regulates vascular smooth muscle cell $Ca^{2+}$ handling. *Journal of Vascular Research* **50** 421–429. (doi:10.1159/000354252)

**Jankovic SM, Varjacic M & Protic B** 2001 Relaxant effect of oxytocin on isolated human oviduct. *Croatian Medical Journal* **42** 511–516.

**Kamm KE & Stull JT** 1985 The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annual Review of Pharmacology and Toxicology* **25** 593–620. (doi:10.1146/annurev.pa.25.040185.003113)

**Kessels MM, Qualmann B, Thole HH & Sierralta WD** 1998 Subcellular localization of estradiol receptor in MCF7 cells studied with nanogold-labelled antibody fragments. *European Journal of Histochemistry* **42** 259–270.

**Kim I, Je HD, Gallant C, Zhan Q, Riper DV, Badwey JA, Singer HA & Morgan KG** 2000 $Ca^{2+}$-calmodulin-dependent protein kinase II-dependent activation of contractility in ferret aorta. *Journal of Physiology* **15** 367–374. (doi:10.1111/j.1469-7793.2000.00367.x)

**Kim HR, Appel S, Vetterkind S, Gangopadhyay SS & Morgan KG** 2008 Smooth muscle signalling pathways in health and disease. *Journal of Cellular and Molecular Medicine* **12** 2165–2180. (doi:10.1111/j.1582-4934.2008.00552.x)

**Kisielewska J, Flint AP & Ziecik AJ** 1996 Phospholipase C and adenylate cyclase signalling systems in the action of hCG on porcine myometrial smooth muscle cells. *Journal of Endocrinology* **148** 175–180. (doi:10.1677/joe.0.1480175)

**Kisielewska J, Zezula-Szpyra A, Bilińska B, Flint AP & Ziecik AJ** 1997 Effect of estradiol and progesterone on growth of porcine myometrial smooth muscle cells and phospholipase C and adenylate cyclase signalling systems *in vitro*. *Folia Histochemica et Cytobiologica* **35** 143–149.

**Kumar P, Wu Q, Chambliss KL, Yuhanna IS, Mumby SM, Mineo C, Tall GG & Shaul PW** 2007 Direct interactions with Gαi and Gβγ mediate nongenomic signaling by estrogen receptor-α. *Molecular Endocrinology* **21** 1370–1380. (doi:10.1210/me.2006-0360)

**Lee DY, Chai YG, Lee EB, Kim KW, Nah SY, Oh TH & Rhim H** 2002 17β-estradiol inhibits high-voltage-activated calcium channel currents in rat sensory neurons via a non-genomic mechanism. *Life Sciences* **70** 2047–2059. (doi:10.1016/S0024-3205(01)01534-X)

**Lin AH, Leung GP, Leung SW, Vanhoutte PM & Man RY** 2011 Genistein enhances relaxation of the spontaneously hypertensive rat aorta by transactivation of epidermal growth factor receptor following binding to membrane estrogen receptors-α and activation of a G protein-coupled, endothelial nitric oxide synthase-dependent pathway. *Pharmacological Research* **63** 181–189. (doi:10.1016/j.phrs.2010.11.007)

**Livak KJ & Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) ($-\Delta\Delta$C) method. *Methods* **25** 402–408. (doi:10.1006/meth.2001.1262)

**Lösel R & Wehling M** 2003 Nongenomic actions of steroid hormones. *Nature Reviews. Molecular Cell Biology* **4** 46–56.

**Lösel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K & Wehling M** 2003 Nongenomic steroid action: controversies, questions, and answers. *Physiological Reviews* **83** 965–1006.

**Marino M, Pallottini V & Trentalance A** 1998 Estrogens cause rapid activation of IP3-PKC-α signal transduction pathway in HEPG2 cells. *Biochemical and Biophysical Research Communications* **245** 254–258. (doi:10.1006/bbrc.1998.8413)

**Marino M, Galluzzo P & Ascenzi P** 2006 Estrogen signaling multiple pathways to impact gene transcription. *Current Genomics* **7** 497–508. (doi:10.2174/138920206779315737)

**Moenter SM & Chu Z** 2012 Glucosensing by GnRH neurons: inhibition by androgens and involvement of AMP-activated protein kinase. *Molecular Endocrinology* **25** 847–858.

**Mohan S, Ahmad AS, Glushakov AV, Chambers C & Doré S** 2012 Putative role of prostaglandin receptor in intracerebral hemorrhage. *Frontiers in Neurology* **3** 1–17. (doi:10.3389/fneur.2012.00145)

**Moore GD & Croxatto HB** 1988*a* Synthetic microspheres transferred to the rat oviduct on day 1 of pregnancy mimic the transport of native ova. *Journal of Reproduction and Fertility* **82** 735–742. (doi:10.1530/jrf.0.0820735)

**Moore GD & Croxatto HB** 1988*b* Effects of delayed transfer and treatment with oestrogen on the transport of microspheres by the rat oviduct. *Journal of Reproduction and Fertility* **83** 795–802. (doi:10.1530/jrf.0.0830795)

**Nadal A, Díaz M & Valverde MA** 2001 The estrogen trinity: membrane, cytosolic, and nuclear effects. *News in Physiological Sciences* **16** 251–255.

**Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Petterson K, Warner M & Gustafsson JA** 2001 Mechanisms of estrogen action. *Physiological Reviews* **81** 1535–1565.

**Orihuela PA, Ríos M & Croxatto HB** 2001 Disparate effects of estradiol on egg transport and oviductal protein synthesis in mated and unmated rats. *Biology of Reproduction* **65** 1232–1237. (doi:10.1095/biolreprod65.4.1232)

**Orihuela PA, Parada-Bustamante A, Cortés PP, Gatica C & Croxatto HB** 2003 Estrogen receptor, cyclic adenosine monophosphate, and protein kinase A are involved in the nongenomic pathway by which estradiol accelerates oviductal oocyte transport in cyclic rats. *Biology of Reproduction* **68** 1225–1231. (doi:10.1095/biolreprod.102.011395)

**Orihuela PA, Parada-Bustamante A, Zúñiga LM & Croxatto HB** 2006 Inositol triphosphate participates in an oestradiol nongenomic signalling pathway involved in accelerated oviductal transport in cycling rats. *Journal of Endocrinology* **188** 579–588. (doi:10.1677/joe.1.06448)

**Orihuela PA, Zuñiga LM, Rios M, Parada-Bustamante A, Sierralta WD, Velásquez LA & Croxatto HB** 2009 Mating changes the subcellular distribution and the functionality of estrogen receptors in the rat oviduct. *Reproductive Biology and Endocrinology* **7** 139. (doi:10.1186/1477-7827-7-139)

**Orihuela PA, Parada-Bustamante A, Cardenas H, Tapia-Pizarro A, Oróstica L, Reuquén P** 2013 Mating effects on female reproductive organs: the paradigm of estrogen signaling pathways in the oviduct. In *Human and Animal Mating* edn 1, pp 1–30. Eds M Nakamara & T Ito. Nova Science Publishers: New York.

**Oróstica ML, Zuñiga LM, Utz D, Parada-Bustamante A, Velásquez LA, Cardenas H & Orihuela PA** 2013 Tumour necrosis factor-α is the signal induced by mating to shutdown a 2-methoxyestradiol nongenomic action necessary to accelerate oviductal egg transport in the rat. *Reproduction* **145** 109–117.

**Oróstica ML, López J, Rojas I, Rocco J, Díaz P, Reuquen P, Cardenas H, Parada-Bustamante A & Orihuela PA** 2014 Estradiol increases cAMP in the oviductal secretory cells through a nongenomic mechanism. *Reproduction* **148** 285–294.

**Ortíz ME, Villalón M & Croxatto HB** 1979 Ovum transport and fertility following postovulatory treatment with estradiol in rats. *Biology of Reproduction* **21** 1163–1167.

**Parada-Bustamante A, Orihuela PA, Ríos M, Navarrete-Gómez PA, Cuevas CA, Velásquez LA, Villalón MJ & Croxatto HB** 2007 Catechol-o-methyltransferase and methoxyestradiols participate in the intraoviductal nongenomic pathway through which estradiol accelerates egg transport in cycling rats. *Biology of Reproduction* **77** 934–941. (doi:10.1095/biolreprod.107.061622)

**Parada-Bustamante A, Orihuela PA, Ríos M, Cuevas CA, Oróstica ML, Velásquez LA, Villalón MJ & Croxatto HB** 2010 A non-genomic signaling pathway shut down by mating changes the estradiol-induced gene expression profile in the rat oviduct. *Reproduction* **139** 631–644. (doi:10.1530/REP-09-0218)

**Parada-Bustamante A, Croxatto HB, Cárdenas H & Orihuela PA** 2012 Differential participation of endothelin receptors in estradiol-induced oviductal egg transport acceleration in unmated and mated rats. *Asian Pacific Journal of Reproduction* **1** 22–26. (doi:10.1016/S2305-0500(13)60042-1)

**Parada-Bustamante A, Valencia C, Reuquen P, Diaz P, Rincon-Rodriguez R & Orihuela PA** 2015 Role of 2-methoxyestradiol, an endogenous estrogen metabolite, in health and disease. *Mini Reviews in Medicinal Chemistry* **15** 427–438. (doi:10.2174/1389557515666150226121052)

**Perez Martinez S, Viggiano M, Franchi AM, Herrero MB, Ortiz ME, Gimeno MF, Villalón M, Perez Martinez S, Viggiano M, Franchi AM *et al.*** 2000 Effect of nitric oxide synthase inhibitors on ovum transport and oviductal smooth muscle activity in the rat oviduct. *Journal of Reproduction and Fertility* **118** 111–117.

**Powis G, Seewald MJ, Gratas C, Melder D, Riebow J & Modest EJ** 1992 Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. *Cancer Research* **52** 2835–2840.

**Qualmann B, Kessels MM, Thole HH & Sierralta WD** 2000 A hormone pulse induces transient changes in the subcellular distribution and leads to a lysosomal accumulation of the estradiol receptor α in target tissues. *European Journal of Cell Biology* **79** 383–393. (doi:10.1078/0171-9335-00061)

**Razandi M, Pedram A, Greene GL & Levin ER** 1999 Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ERβ expressed in Chinese hamster ovary cells. *Molecular Endocrinology* **13** 307–319.

**Reynolds ES** 1963 The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17** 208–212.

**Rhee SG** 2001 Regulation of phosphoinositide-specific phospholipase C. *Annual Review of Biochemistry* **70** 281–312. (doi:10.1146/annurev.biochem.70.1.281)

**Ríos M, Hermoso M, Sánchez TM, Croxatto HB & Villalón MJ** 2007 Effect of oestradiol and progesterone on the instant and directional velocity of microsphere movements in the rat oviduct: gap junctions mediate the kinetic effect of oestradiol. *Reproduction, Fertility, and Development* **19** 634–640.

**Sawai T, Bernier F, Fukushima T, Hashimoto T, Ogura H & Nishizawa Y** 2002 Estrogen induces a rapid increase of calcium-calmodulin-dependent protein kinase II activity in the hippocampus. *Brain Research* **20** 308–311. (doi:10.1016/S0006-8993(02)03186-4)

**Singh S & Gupta PD** 1997 Induction of phosphoinositide-mediated signal transduction pathway by 17β-oestradiol in rat vaginal epithelial cells. *Journal of Molecular Endocrinology* **19** 249–257. (doi:10.1677/jme.0.0190249)

**Szóstek AZ, Siemieniuch MJ, Deptula K, Woclawek-Potocka I, Majewska M, Okuda K & Skarzynski DJ** 2011 Ovarian steroids modulate tumor necrosis factor-α and nitric oxide-regulated prostaglandin secretion by cultured bovine oviductal epithelial cells. *Domestic Animal Endocrinology* **41** 14–23.

**Townsend EA, Thompson MA, Pabelick CM & Prakash YS** 2010 Rapid effects of estrogen on intracellular $Ca^{2+}$regulation in human airway smooth muscle. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **298** L521–L530. (doi:10.1152/ajplung.00287.2009)

**Townsend EA, Sathish V, Thompson MA, Pabelick CM & Prakash YS** 2012 Estrogen effects on human airway smooth muscle involve cAMP and protein kinase A. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **303** L923–L928. (doi:10.1152/ajplung.00023.2012)

**Turner CD** 1961 Endocrinology of the ovary. In *General Endocrinology*. Ed CD Turner, 3rd ed pp 365–409. Philadelphia: WB Saunders Company.

**Watson CS & Gametchu B** 2003 Proteins of multiple classes may participate in nongenomic steroid actions. *Experimental Biology and Medicine* **228** 1272–1281.

**Watson CS, Jeng YJ, Hu G, Wozniak A, Bulayeva N & Guptarak J** 2012 Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-α interactions with G protein-αi and caveolin I. *Steroids* **77** 424–432. (doi:10.1016/j.steroids.2011.12.025)

**Watson CS, Hu G & Paulucci-Holthauzen AA** 2014 Rapid actions of xenoestrogens disrupt normal estrogenic signaling. *Steroids* **81** 36–42. (doi:10.1016/j.steroids.2013.11.006)

**Wehling M, Schultz A & Lösel R** 2006 Nongenomic actions of estrogens: exciting opportunities for pharmacology. *Maturitas* **54** 321–326. (doi:10.1016/j.maturitas.2006.04.021)

**Wijayagunawardane MP & Miyamoto A** 2004 Tumor necrosis factor α system in the bovine oviduct: a possible mechanism for embryo transport. *Journal of Reproduction and Development* **50** 57–62. (doi:10.1262/jrd.50.57)

**Wijayagunawardane MP, Gabler C, Killian G & Miyamoto A** 2003 Tumor necrosis factor α in the bovine oviduct during the estrous cycle: messenger RNA expression and effect on secretion of prostaglandins, endothelin-1, and angiotensin II. *Biology of Reproduction* **69** 1341–1346. (doi:10.1095/biolreprod.103.017327)

**Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM & Shaul PW** 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Gα(i). *Journal of Biological Chemistry* **276** 27071–27076. (doi:10.1074/jbc.M100312200)

**Yang YR, Follo MY, Cocco L & Suh PG** 2013 The physiological roles of primary phospholipase C. *Advances in Biological Regulation* **53** 232–241. (doi:10.1016/j.jbior.2013.08.003)

**Zhu X & Birnbaumer L** 1996 G protein subunits and the stimulation of phospholipase C by Gs-and Gi-coupled receptors: Lack of receptor selectivity of Gα(16) and evidence for a synergic interaction between Gβ γ and the α subunit of a receptor activated G protein. *PNAS* **93** 2827–2831. (doi:10.1073/pnas.93.7.2827)

Received 25 March 2015
First decision 24 April 2015
Revised manuscript received 6 July 2015
Accepted 9 July 2015