Inositol triphosphate participates in an oestradiol nongenomic signalling pathway involved in accelerated oviductal transport in cycling rats

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Abstract

Oestradiol (E2) accelerates oviductal transport of oocytes in cycling rats through a nongenomic pathway that involves the cAMP–PKA signalling cascade. Here we examined the role of the inositol triphosphate (IP3) and mitogen-activated protein kinase (MAPK) signalling cascades in this nongenomic pathway. Oestrous rats were injected with E2 s.c. and intrabursally (i.b) with the selective inhibitors of phospholipase C (PLC) ET-18-OCH3 or MAPK PD98059. The number of eggs in the oviduct assessed 24 h later showed that ET-18-OCH3 blocked E2-induced egg transport acceleration, whereas PD98059 had no effect. Other oestrous rats were treated with E2 s.c. and 1, 3 or 6 h later oviducts were excised and the levels of IP3 and phosphorylated MAPK p44/42 (activated) were determined by radioreceptor assay and western blot, respectively. Oestradiol administration increased IP3 level at 1 and 6 h after treatment, whereas activated MAPK p44/42 level was unchanged. Finally, we explored whether cAMP-PKA and PLC-IP3 signalling cascades are coupled. Inhibition of adenylyl cyclase by i.b. injection of SQ 22536 blocked the increase of IP3 levels induced by E2, while inhibition of PLC by ET-18-OCH3 had no effect on E2-induced PKA activity. Furthermore, activation of adenylyl cyclase by Forskolin increased oviductal IP3 levels. Thus, activation of PLC-IP3 by E2 requires previous stimulation of cAMP-PKA. We conclude that the nongenomic pathway utilised by E2 to accelerate oviductal transport of oocytes in cycling rats involves successive activation of the cAMP-PKA and PLC-IP3 signalling cascades and does not require activation of MAPK. These findings clearly illustrate a nongenomic pathway triggered by E2 that regulates a complex physiologic process accomplished by an entire organ.


Introduction

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 oestradiol (E2) on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 h to less than 24 h (Ortiz et al. 1979).

The classical model of E2 action on its target cells comprises its binding to oestrogen receptors (ER), leading to modification of gene expression and protein synthesis (Nilsson et al. 2001). However, this model cannot explain the E2 effects that are not blocked by inhibitors of transcription or translation, or are too rapid to be due to changes in gene expression. Therefore, these effects have been named nongenomic (Lössel & Wheling 2003, Lössel et al. 2003). The nongenomic actions of E2 often involve the generation of intracellular secondary messengers, and various signal transduction cascades upon binding to ER (Nadal et al. 2001).

Activation of signal transduction cascades by E2 modulates diverse downstream pathways that have discrete cellular actions, including stimulation of adenylyl cyclase in breast and vascular tissues (Aronica et al. 1994, Farhat et al. 1996) and phospholipase C (PLC) type β in rat osteoblasts (Le Mellay et al. 1997), generation of inositol triphosphate (IP3) in rat vaginal epithelial cells (Singh & Gupta 1997), activation of Ca2+ flux and Ca2+-activated K+ channels in arterial smooth muscle (Prakash et al. 1999, Valverde et al. 1999), activation of cGMP-dependent protein kinase in pancreatic β-cells (Ropero et al. 1999), activation of protein kinases A (PKA) and C (PKC) in β-endorphin neurons (Qu et al. 2003) and protein phosphorylation via activation of tyrosine kinases, phosphatidylinositol 3 kinase (PI3K) and mitogen-activated protein kinases’ (MAPK) pathways in MCF-7

We previously demonstrated that RNA and protein synthesis inhibitors block E2-induced oviductal embryo transport acceleration in pregnant rats, but failed to do so in cyclic rats (Ríos et al. 1997, Orihuela et al. 2001). Furthermore, in cyclic rats exogenous E2 activates protein phosphorylation in the oviduct via a nongenomic action, since such activation occurs when mRNA synthesis is completely suppressed by α-amanitin (Orihuela & Croxatto 2001). Oestradiol-induced phosphorylation is essential for its effect on oocyte transport in cycling rats; local administration of a broad-spectrum inhibitor of protein kinases totally blocks E2-induced acceleration of egg transport (Orihuela & Croxatto 2001, Parada-Bustamante et al. 2003). Moreover, local administration of the anti-oestrogen ICI 182780 and of a selective inhibitor of adenylyl cyclase SQ22536 totally blocks egg transport acceleration induced by E2, whereas a PKA inhibitor Rp-cAMP only partially blocks this effect of E2 (Orihuela et al. 2003). In addition, the adenylyl cyclase activator Forskolin mimics the effect of E2 on egg transport (Orihuela et al. 2003). Thus, E2 accelerates oviductal transport of oocytes via a nongenomic action that involves oviductal protein phosphorylation. The nongenomic pathway by which E2 accelerates oviductal egg transport in the rat requires participation of ER, generation of cAMP and activation of PKA.

In this work, we explored the contribution of IP3 and MAPK signalling cascades in this nongenomic pathway. First we examined the role of IP3 and MAPK on accelerated oocyte transport induced by E2. The effect of E2 on the levels of IP3 or phosphorylated MAPK p44/42 in the rat oviduct was then determined. Finally, we determined whether cAMP-PKA and PLC-IP3 signalling cascades are coupled. For this purpose, we examined the effect of a selective inhibitor of adenylyl cyclase or PLC on E2-induced increase of IP3 levels or PKA activity, respectively. In addition, we determined the effect of an activator of adenylyl cyclase on oviductal IP3 level.

**Materials and Methods**

**Animals**

Sprague-Dawley rats (bred in house) 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. The phases of the oestrous cycle were determined by daily vaginal smears (Turner 1961). Only rats that showed at least two regular 4-day cycles were used. The day of oestrus was considered day 1 of the cycle. The care and manipulation of the animals was made in accordance with the ethical guidelines of our institution.

**Treatments**

**Systemic administration of drugs** On day 1 of the cycle E2 1 µg in 0·1 mL of propylene glycol or the PLC inhibitor ET-18-OCH3 (1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine; Calbiochem, La Jolla, CA, USA; Powis et al. 1992) 32 µg in 0·1 mL of saline were injected s.c. as a single dose. Control rats received the corresponding vehicle alone.

**Local administration of drugs** The drugs described below were injected into each ovarian bursa dissolved in the appropriate vehicle (control rats received the corresponding vehicle alone):

- Adenylyl cyclase inhibitor: SQ 22536 (9-(tetrahydro-2′furyl) adenine; Calbiochem; Goldsmith & Abrams 1991) was injected as a single dose of 30 µg in 4 µL of saline solution. Previously, we have shown that this concentration completely blocks the E2-induced oviductal egg transport acceleration (Orihuela et al. 2003).
- MAPK inhibitor: PD98059 (2-(2-Hydroxyethylamino)-6-benzylamino-9-methylpurine; Calbiochem; Verin et al. 2000, Xiao et al. 2004) was injected as a single dose of 0·5, 5, 50 µg in 4 µL of 0·1% DMSO.
- Phospholipase C inhibitor: ET-18-OCH3 (1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine; Calbiochem; Powis et al. 1992) was injected as a single dose of 0·032, 0·32, 3·2 or 32 µg in 4 µL saline solution.
- Adenylyl cyclase activator: Forskolin (β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one; Sigma Chemical Co.; Uneyama et al. 1993) was injected as a single dose at a concentration of 20 µg in 4 µL 0·1% DMSO.

**Animal surgery**

Intrabursal administration of drugs was performed in the morning of day 1 of the cycle as described by Orihuela et al. (2001). At this time, ovulation has already taken place, so this treatment cannot affect the number of oocytes ovulated.

**Assessment of egg transport**

Twenty-four hours after treatment, animals were sacrificed and their oviducts were flushed individually with saline. Flushings were examined under low-power magnification (× 25). The number of eggs in the oviduct was recorded. We have previously determined from egg recovery experiments from uterus and vagina and from placing ligatures in the uterine horns that the reduction in the number of oviductal oocytes following treatment with E2 corresponds to premature transport to the uterus (Ortiz et al. 1979). Thus, we refer to it as E2-induced accelerated egg transport.

**Measurement of IP3 levels**

Whole oviducts in groups of four were homogenised in 0·25 mL ice cold 1 M trichloroacetic acid (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 comprising of a mixture of 1,1,2-Trichloro-trifluoroethane (TCTFE; Sigma) and Trioctylamine (Sigma), 3:1 (v/v). Levels of IP3 were determined using Inositol-1,4,5-Trisphosphate \([^{3}H]\) Radioreceptor Assay Kit (catalogue number NEK064 (NEN Life Science Products, Boston, MA, USA). This assay is based on 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.

MAPK p44/42 phosphorylation

Polyclonal antibodies that recognize the phosphorylated state of MAPK on Tyr204 (anti-phosphoMAPK p44/42, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or total MAPK (anti-MAPK p42, Santa Cruz Biotechnology) were used to assess activation of MAPK. Whole oviducts were removed and cleaned from fat tissue. Because we wanted to analyse only oviductal proteins, oviducts were flushed with saline in order to remove the cumulus-oocytes complex and thus avoid contamination with their proteins. Then, oviducts in groups of four (obtained from two rats) were homogenized on ice for 10 sec in 1 mL of buffer containing 0.25 mM sucrose, 3.0 mM MgCl\(_2\), 25 mM Tris, and 0.5 mM phenylmethylsulfonyl fluoride (Taragnat 1988) followed by centrifugation at 6000 r.p.m. for 10 min at 4 °C. The supernatant (clarified homogenate) was harvested and stored at −20 °C until used. The protein concentration in the clarified homogenate was determined according to Bradford (1976) using BSA as standard. Aliquots of the clarified homogenate containing 100 µg protein were denatured for 2 min at 90 °C in equal volume of 0.125 M Tris–HCl (pH 6.8) containing 4% SDS, 10% \(\beta\)-mercaptoethanol, 20% glycerol and 0.04% bromophenol blue. Samples were run on 12% SDS polyacrylamide slab gels according to the method of Laemmli (1970) utilizing a mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electroblotted onto nitrocellulose membranes (Bio-Rad; Towbin et al. 1979). Nitrocellulose blots were blocked by incubation overnight at 4 °C in TTBS (100 mM Tris/HCl (pH 7.5), 0.9% v/v 150 mM NaCl and 0.05% v/v Tween 20) containing 1% BSA and were incubated for 2 h with a rabbit anti-MAPK p44 or anti-phosphoMAPK p44/42 in 1:200 dilution. Blots were rinsed five times for 5 min each in TBS (100 mM Tris/HCl (pH 7.5), and 0.9% v/v 150 mM NaCl) and were incubated for 2 h in TTBS containing 1:5000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Chemicon International, Temecula, CA, USA). The horseradish peroxidase activity was detected by enhanced chemiluminescence using western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA). Negative controls consisting of oviductal samples without anti-MAPK p44 or anti-phosphoMAPK p44/42 were included. Protein extracts of a breast cancer cell line (MCF-7, kindly donated by Gareth Owen, Pontificia Universidad Católica de Chile, Santiago, Chile) were used as positive controls.

Activity of PKA

Whole oviducts in groups of four were sonicated in sample preparation buffer (50 mM Tris–Hcl, 50 mM \(\beta\)-mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM PMSE, 10 mM Benzamidine (pH 7.5)) and centrifuged for 5 min at 10000 \(g\). Aliquots of the supernatant (100 µg) were used to determine PKA activity with a Protein Kinase Assay Kit, Non-Radioactive, catalogue no. 538484, lot number B31285 (Calbiochem). This kit provides an enzyme-linked immunosorbent assay that utilizes a synthetic PKA substrate peptide and a monoclonal biotinylated antibody that recognizes the phosphorylated form of the peptide. Colour was developed with peroxidase-conjugated streptavidin and o-phenylenediamine as substrate. Optical density was read at 492 nm, with a microplate reader (BIO-TEK Instruments Inc., Winooski, VT, USA). A standard curve using 1–100 mU bovine heart PKA (Sigma; specific activity: 1–2 units/µg protein) was constructed to determine the PKA activity in the unknown samples. Although we used bovine heart PKA instead of rat PKA the homology between these enzymes is highly conserved (Titani et al. 1984, Kuno et al. 1987), therefore we extrapolated data obtained from the bovine PKA to our rat model.

Activity of MAPK

Whole oviducts in groups of four were homogenized in RIPA buffer (50 mM Tris–Hcl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM DDT, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS and protease inhibitors cocktail) and centrifuged for 10 min at 12000 \(g\). Aliquots of the supernatant (150 µg) were used to determine MAPK activity with a MAP Kinase (ERK1/2) Activity Assay Kit, catalogue number SGT415, lot number 0606002744 (Chemicon). This kit provides an enzyme-linked immunosorbent assay that utilizes a biotinylated synthetic MAPK substrate peptide (myelin basic protein – MBP). The fraction of phosphorylated substrate is visualized using a phospho–MBP monoclonal antibody and a horseradish peroxidase conjugated secondary antibody and an ensuing chromogenic substrate reaction. Optical density was read at 450 nm with a microplate reader (BIO-TEK Instruments Inc.).
Statistical Analysis

The results are presented as mean ± s.e.m. Overall analysis was done by Kruskal-Wallis test, followed by Mann-Whitney test for pair-wise comparisons when overall significance was detected. The actual number of replicates (n) in experiments done to determine IP3 levels, MAPK and PKA activity in the rat oviduct corresponds to three or four replicates. In each replicate, two rats for each treatment group were used and the four oviducts of these two animals were homogenised as a pool to determine IP3 levels and PKA activity. The actual n in experiments done to determine the effect of drugs on oviductal egg transport is the total number of rats used in each experimental group, because the total number of eggs recovered from the two oviducts of a single rat corresponds to a single data point.

Results

Effect of local treatment with specific signalling pathway inhibitors on E2-induced egg transport acceleration

Two experiments were performed to determine whether local administration of selective MAPK (PD98059) or PLC-IP3 (ET-18-OCH3) signalling inhibitors can prevent the acceleration of oviductal egg transport induced by E2 in cycling rats. A total of 60 animals on day 1 of the cycle were used and, for each experiment, they were divided into the following treatment groups: 1) vehicle plus propylene glycol; 2) PD98059 (50 µg) or ET-18-OCH3 (32 µg) plus propylene glycol; 3) vehicle plus E2 and 4) PD98059 (0.5, 5 or 50 µg) or ET-18-OCH3 (0.032, 0.32, 3.2 or 32 µg) plus E2. Twenty-four hours after treatment, egg transport was assessed as described. Replicas of this experiment are stated in Figure 1.

The results are shown in Figure 1. The mean number of eggs recovered from the oviducts of control groups ranged from 9.3 ± 1.3 to 8.3 ± 0.7, while in groups treated with E2 it ranged from 0.8 ± 0.3 to 0.5 ± 0.2. Local administration of inhibitors alone did not affect oviductal egg recovery (range 8.2 ± 1.1 to 7.9 ± 1.3). The PLC inhibitor blocked the E2-induced oviductal egg transport acceleration in a dose-dependent manner (1.0 ± 0.6 to 9.0 ± 0.7) while the MAPK inhibitor had no effect (range 1.0 ± 0.7 to 2.2 ± 1.7).

Effect of systemic administration of ET-18-OCH3 on E2-induced egg transport acceleration

Since local administration of ET-18-OCH3 inhibited the effect of E2 on egg transport, this experiment was done to rule out that the effect was systemic rather than local. A total of 11 rats were divided into the following treatment groups: 1) vehicle plus propylene glycol; 2) vehicle plus E2 and 3) ET-18-OCH3 32 µg plus E2. Twenty-four hours after treatment, egg transport was assessed as described.

The mean number of eggs recovered from the oviducts of control group was of 8.6 ± 1.1 (n = 4) while in the group treated with E2 it was 1.5 ± 0.7 (n = 3). In the group treated with ET-18-OCH3 plus E2 the mean number of eggs was 2.5 ± 1.9 (n = 4).

Effect of ET-18-OCH3 on IP3 level in the oviduct

Here we verified that intrabursal administration of ET-18-OCH3 effectively inhibited oviductal IP3 levels. A total of 16 rats were injected with ET-18-OCH3 32 µg into one ovarian bursa and with saline in the contralateral bursa, and 6 h later oviducts were excised and processed to determine IP3 levels as described.

The level of IP3 was decreased in the oviducts treated with PLC inhibitor (0.08 ± 0.01 pmol/oviduct, n=4) but not in the contralateral oviduct treated with saline (0.9 ± 0.1 pmol/oviduct, n = 4).

Effect of PD98059 on MAPK activity in the oviduct

Here we verified that intrabursal administration of PD98059 effectively inhibited oviductal MAPK activity. A total of 16 rats were injected with PD98059 50 µg into one ovarian bursa and with saline in the contralateral bursa, and 6 h later oviducts were excised and processed to determine MAPK activity as described.

The MAPK activity was decreased in the oviducts treated with MAPK inhibitor (0.09 ± 0.02 OD 450 nm, n=4) but not in the contralateral oviduct treated with saline (0.60 ± 0.07 OD 450 nm, n = 4).

Effect of E2 on IP3 levels in the oviduct

This experiment determined whether E2 administration increases IP3 levels in the oviduct. A total of 36 animals on day 1 of the cycle were treated with vehicle or E2 and 1, 3 or 6 h after treatment their oviducts were excised and processed to determine IP3 levels as described. This experiment consisted of 3 replicas.

The basal level of IP3 in the oviduct ranged from 0.30 ± 0.04 to 0.60 ± 0.08 pmol/oviduct. Oestradiol administration increased 10–12 times the IP3 levels measured at 1 and 6 h after treatment, but had no effect at 3 h (Figure 2).

Effect of E2 on MAPK phosphorylation in the oviduct

This experiment determined whether E2 administration affects the level of phosphorylated MAPK p44/42 in the oviduct. A total of 16 animals on day 1 of the cycle were treated with E2 and 0, 1, 3 or 6 h after treatment their
Oviducts were excised and processed to determine the level of phosphorylated MAPK p44/42 as described. This experiment consisted of two replicas. Figure 3 shows that immunoblots of phosphorylated MAPK p44/42 of whole oviducts did not reveal any specific band compared with positive controls, although total MAPK p42 was present.

Effect of E2 and/or ET-18-OCH3 on oviductal PKA activity

Here we determined whether exogenous E2 and/or inhibition of PLC-IP3 cascade affects oviductal PKA activity. A total of 28 animals on day 1 of the cycle were divided into four treatment groups: 1) vehicle plus propylene glycol; 2) ET-18-OCH3 32 µg i.b. plus propylene glycol; 3) vehicle plus E2 and 4) ET-18-OCH3 plus E2. Previously we have shown that E2 increases cAMP levels in rat oviducts at 3 h after treatment (Orihuela et al. 2003). Therefore, in this experiment PKA activity in the oviduct was assessed at 3·5 h after treatment as described. Replicas of this experiment are stated in Figure 4. In order to confirm that PLC-IP3 signalling was inhibited following treatment with PLC inhibitor and E2, another 12 animals were divided into two treatment groups: 1) vehicle plus propylene glycol and 2) ET-18-OCH3 plus E2, and 3·5 h later oviductal IP3 level was measured as described.
The results are shown in Figure 4. The mean PKA activity (± s.e.) in oviducts of the control group was 10.8 ± 0.2 mU/µg protein, while in the group treated with E2 it was 46.1 ± 10.2 mU/µg protein. Local administration of the PLC inhibitor affected neither basal PKA activity (13.4 ± 1.5 mU/µg protein) nor the E2-induced PKA increase (32.3 ± 5.9 mU/µg protein) although it caused a 5-fold decrease in IP3 levels (vehicle+propylene glycol, 1.1 ± 0.2 pmol/oviduct; ET-18-OCH₃+E2, 0.02 ± 0.02 pmol/oviduct, n=3).

Effect of specific cAMP-PKA signalling pathway inhibitor (SQ22536) on oviductal IP3 levels after E2 administration

Here we assessed whether inhibition of cAMP-PKA signalling cascade affects the level of IP3 in the oviduct following treatment with E2. A total of 24 animals on day 1 of the cycle were divided into four treatment groups: 1) vehicle plus propylene glycol; 2) SQ22536 30 µg i.b. plus propylene glycol; 3) vehicle plus E2 and 4) SQ22536 plus E2. Six hours after treatment, IP3 levels in the oviduct were assessed as described. This experiment consisted of four replicas.

The results are shown in Figure 5. The IP3 levels in the oviducts of the control group were 0.80 ± 0.08 pmol/oviduct, while in the group treated with E2 they were 5.20 ± 0.07 pmol/oviduct. Local administration of the adenyl cyclase inhibitor did not affect basal IP3 levels (0.80 ± 0.09 pmol/oviduct), although it completely blocked the E2-induced IP3 increase (1.30 ± 0.86 pmol/oviduct).

Effect of specific cAMP-PKA signalling pathway activator (Forskolin) on oviductal IP3 levels

Here we assessed whether activation of cAMP-PKA cascade affects the level of IP3 in the oviduct. A total of
Discussion

Oestrogens elicit a variety of cellular responses through genomic and nongenomic pathways. Previously, we demonstrated that E₂ accelerates oviductal oocyte transport in cycling rats through a nongenomic pathway, which requires activation of cAMP-PKA signalling cascade (Orihuela & Croxatto 2001, Orihuela et al. 2003). Here, we report that PLC-IP₃ is a functional component of this E₂ nongenomic pathway, which is located downstream of the cAMP-PKA signalling cascade.

IP₃ levels increased in the rat oviduct following E₂ treatment, suggesting that PLC activation is involved in accelerated oviductal transport induced by E₂. Indeed blockade of PLC by ET-18-OCH₃ suppressed the E₂-induced egg transport acceleration in a dose-dependent manner. These findings indicate unequivocally that PLC-IP₃ signalling cascade is another component in the nongenomic pathway by which E₂ accelerates egg transport. This is in keeping with findings in other organs or cell lines. It has been documented that an increase in the turnover of inositol lipids is one mechanism used by E₂ to elicit nongenomic actions (Lössel et al. 2003). The initial event is the hydrolysis of phosphatidylinositol 4,5-bisphosphate, a reaction catalyzed by a phosphoinositide-specific PLC, which generates two intracellular secondary messengers, IP₃ and 1,2-diacylglycerol. Binding of IP₃ to specific receptors on the endoplasmic reticulum mobilises intracellular calcium, which results in increased phosphorylation of proteins (Martin 1991). In female rat osteoblasts, E₂ causes a 5–60 fold increase in the concentration of intracellular Ca²⁺ by activation of PLC-β and subsequent formation of IP₃ (Le Mellay 1997). Singh and Gupta (1997) have shown that E₂ increases the metabolism of phosphoinositol lipids as well as production of inositol phosphates in rat vaginal epithelial cells independently of mRNA and protein synthesis. On the other hand, oestrogen addition to a hepatoma cell line HEPG2 causes rapid increase of IP₃ production (Marino 1998). Our findings show that a nongenomic action of E₂ mediated by PLC-IP₃ is also present in the mammalian oviduct. Oestradiol increased oviductal IP₃ levels at 1 and 6 h but not at 3 h after administration. This oscillating pattern suggests that the first increase is a direct response to E₂ and the second increase is in response to downstream intracellular changes. Alternatively, the two increases may be taking place each one in different cell types, e.g. epithelium and muscle.

Phospholipase C activation also produces 1,2-diacylglycerol with subsequent increased PKC activity (Martin 1991). Furthermore, PKC activation is necessary for E₂-induced oviductal protein phosphorylation (Orihuela & Croxatto 2001). However, we have previously demonstrated that specific PKC inhibitors did not block the E₂-induced egg transport acceleration (Orihuela et al. 2003), indicating that PLC-PKC signalling pathway is not involved in the nongenomic pathway by which E₂ accelerates oviductal egg transport.

The MAPK family is a conserved network of signal transduction enzymes that are activated by phosphorylation (Cobb & Goldsmith 1995). Nongenomic activation of MAPK by E₂ has been reported in several cell systems (Chaturvedi & Sarkar 2004, Sengupta et al. 2004, Wade & Dorsa 2003). However, we found that E₂ did not activate MAPKp44/42 and that inhibition of MAPK activity by PD98059 did not block the effect of E₂ on egg transport. These results indicate that the MAPK signalling pathway is not regulated by E₂ in the rat oviduct and that this cascade is not involved in the effect of E₂ on oviductal transport in cycling rats.

With the purpose of demonstrating the efficacy of PLC and MAPK inhibitors in our experimental design, we also measured IP₃ levels and MAPK activity in the rat oviduct after intrabursal administration of ET-18-OCH₃ and PD98059, respectively. Our results showed that IP₃ levels and MAPK activity were decreased in oviducts treated with inhibitors, but not in the contralateral oviduct treated with saline. In addition, we determined that intrabursal administration of ET-18-OCH₃ in a dose that completely blocked E₂-induced egg transport acceleration was unable to exert this effect when it was administered systemically. All these results clearly demonstrate that these inhibitors...
effectively blocked the PLC-IP3 and MAPK signalling cascades in the oviduct and that their effects given intrabursally were due to local rather than systemic action.

We found that inhibition of cAMP-PKA signalling pathway by SQ 22536, at a concentration that blocks the E2-induced egg transport acceleration (Orihuela et al. 2003), blunted the IP3 level increase induced by E2, while inhibition of PLC-IP3 signalling pathway by ET-18-OCH3 had no effect on the E2-induced PKA activity. Furthermore, activation of adenylyl cyclase by Forskolin, at a concentration that accelerates oviductal egg transport (Orihuela et al. 2003) increased oviductal IP3 levels. Thus, activation of PLC-IP3 by E2 requires previous stimulation of cAMP-PKA, showing that both pathways are coupled in a directional sequence. Interaction between cAMP-PKA and PLC-IP3 signalling pathways, in response to neurotransmitters, prostaglandins or neuropeptides has been identified in nonvascular smooth muscle (for review see Abdel-Latif 2001), submandibular acinar cells (Martinez & Zhang 1998) or in osteoblast-like MC3T3-E1 cells (Kozawa et al. 1992). According with our previous results (Orihuela & Croxatto 2001, Orihuela et al. 2001, 2003) and those shown in this paper we propose a model indicating that the nongenomic pathway utilised by E2 to accelerate oviductal transport of oocytes in cycling rats involves successive activation of the cAMP-PKA and PLC-IP3 signalling cascades (Figure 6). Most of the literature describing the nongenomic actions of E2 is limited to report changes in the regulation of specific molecules such as MAPK, IP3, AMPc, etc. in specific cell phenotypes, without clear linkage to physiological processes (reviewed in Lössel & Wehling 2003). However, the evidence presented here highlights a nongenomic action of E2 that is crucial in regulating a complex physiological process accomplished by an entire organ composed of diverse cell phenotypes.

In summary we conclude that PLC-IP3, but not MAPK, signalling pathway is involved in E2-induced egg transport acceleration and that a cAMP-PKA-PLC-IP3 cascade mediates the nongenomic action of E2 in the rat oviduct.

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References

Abdel-Latif AA 2001 Cross talk between cyclic nucleotides and 
phosphoinositide hydrolysis, protein kinases, and contraction in 
Aronica SM, Krauss WL & Katzenellenbogen BS 1994 Estrogen 
action via the cAMP signaling pathway: stimulation of adenylate 
cyclase and cAMP-regulated gene transcription. PNAS 91 8517–8521.
Bradford MM 1976 A rapid and sensitive method for the quantitation 
of microgram amounts of protein utilizing the principle of protein–dye 
Cobb MH & Goldsmith EJ 1995 How MAP kinases are regulated. 
Goldsmith BA & Abrams TW 1991 Reversal of synaptic depression 
by serotonin at Aplysia sensory neuron synapses involves activation 
of adenyl cyclase. PNAS 88 9021–9025.
Cross-talk regulation between cyclic AMP production and 
phosphoinositide hydrolysis induced by prostaglandin E2 in 
Kuno T, Ono Y, Hirai M, Hashimoto S, Shuntoh H & Tanaka C 
1987 Molecular cloning and cDNA structure of the regulatory 
subunit of type I CAMP-dependent protein kinase from rat brain. 
Biochemical Biophysics Research Communication 146 878–883.
Laemmli HK 1970 Cleavage of structural proteins during the assembly 
Le Mellay V, Grosse B & Lieberherr M 1997 Phospholipase C β and 
membrane action of calcitriol and estradiol. Journal of Biological 
Chemistry 272 11902–11907.
Lösel RM, Keschel MR & Wehling M 2003 Nongenomic actions of steroid 
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 & Treantale A 1998 Estrogens cause rapid 
activation of IP3-PKC-alpha signal transduction pathway in 
Márquez DC & Pietras RJ 2001 Membrane-associated binding sites for 
estrogen contribute to growth regulation of human breast 
cancer cells. Oncogene 20 5420–5430.
Martin TF 1991 Receptor regulation of phosphoinositidase C. 
Martinez JR & Zhang GH 1998 Cross-talk in signal transduction 
pathways of rat submandibular acinar cells. European Journal of 
Endocrinology 139 190–193.
Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, 
Lombardi M, Barone MV, Amedrano D, Zannini MS, Abbondance C et al. 2000 
Steroid-induced androgen receptor – oestriadiol receptor 
β-Src complex triggers prostate cancer cell proliferation. 
EMBO Journal 19 5406–5417.
Remacle AS, Mikeli S, Treuter E, Tujague M, Thomsen J, Andersson 
G, Enmark E, Petterson K, Warner M & Gustafsson JA 2001 
Orhiuula PA & Crozatto HB 2001 Acceleration of oviductal transport 
of oocytes induced by estradiol in cycling rats is mediated by 
nongenomic stimulation of protein phosphorylation in the oviduct. 
Biology of Reproduction 65 1238–1245.
Orhiuula PA, Rios M & Crozatto HB 2001 Disparate effects of 
estriadiol on egg transport and oviductal protein synthesis in mated 
Orhiuula PA, Parada-Bustamante A, Cortés PP, Gatica C & Crozatto HB 
2003 Estrogen receptor, cyclic adenosine monophosphate, and 
protein kinase A are involved in the nongenomic pathway by which 
estriadiol accelerates oviductal oocyte transport in cyclic rats. 
Biology of Reproduction 68 1225–1231.
Ortiz ME, Villalón M & Crozatto HB 1997 Ovum transport and 
fertility following postovulatory treatment with estradiol in rats. 
Parada-Bustamante A, Orhiuula PA & Crozatto HB 2003 Effect of 
intruterine insemination with spermatooza or foreign protein on the 
mechanism of action of oestriadiol in the rat oviduct. 
Reproduction 125 677–682.
Prikash YS, Togayabaya AA, Kannan MS, Miller VM, Fitzpatrick LA 
& Sieck GC 1999 Estrstogen increases Ca2+ efflux from female 
porcine coronary arterial smooth muscle. American Journal of 
Physiology 45 1926–1934.
Selective inhibition of phosphatidylinositol phospholipase C 
Qui J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK 
& Kelly MJ 2003 Rapid signaling of estrogen in hypthalamic 
neurons involves a novel G-protein–coupled estrogen receptor that 
Rios M, Orihuela PA & Crozatto HB 1997 Intraovoidal 
administration of ribonucleic acid from estrogen-treated rats mimics 
the effect of estrogen on ovum transport. Biology of Reproduction 
62 279–283.
Ropero AB, Fuentes E, Rovira JM, Ripoll C, Soria B & Nadal A 1999 
Non-genomic actions of 17β-oestradiol in mouse pancreatic 
β-cells are mediated by a cGMP-dependent protein kinase. Journal 
of Physiology 521 397–407.
Sengupta K, Banerjee S, Saxena NK & Banerjee SK 2004 
Thombospondin-1 disrupts estrogen-induced endothelial cell 
proliferation and migration and its expression is suppressed by 
Singh S & Gupta PD 1997 Induction of phosphoinositide-mediated 
signal pathway by 17β-oestradiol in rat vaginal epithelial cells. 
Journal of Molecular Endocrinology 19 249–257.
Stoica GE, Franke TF, Wellstein A, Czubayko F, list HJ, Reiter R, 
Morgan E, Martin MB & Stoica A 2003 Estradiol rapidly activates 
Akt via the ErB2 signaling pathway. Molecular Endocrinology 
17 818–830.
Taragnat C, Berger M & Jean CL 1988 Preliminary characterization, 
androgen-dependence and ontogeny of an abundant protein from 
Titani K, Sasagawa T, Ericsson LH, Kumar S, Smith SB, Krebs EG 
& Walsh KA 1984 Amino acid sequence of the regulatory subunit 
of bovine type 1 adenosine cyclic 3’.5’-phosphate dependent protein 
Turner CD 1961 Endocrinology of the ovary. In General 
USA: WB Saunders Company.


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