

Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase C δ

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Abstract

The gonadotropin hypothesis proposes that elevated serum gonadotropin levels may increase the risk of epithelial ovarian cancer (EOC). We have studied the effect of treating EOC cell lines (OV207 and OVCAR-3) with FSH or LH. Both gonadotropins activated the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and increased cell migration that was inhibited by the MAPK 1 inhibitor PD98059. Both extra- and intracellular calcium ion signalling were implicated in gonadotropin-induced ERK1/2 activation as treatment with either the calcium chelator EGTA or an inhibitor of intracellular calcium release, dantrolene, inhibited gonadotropin-induced ERK1/2 activation. Verapamil was also inhibitory, indicating that gonadotropins activate calcium influx via L-type voltage-dependent calcium channels. The cAMP/protein kinase A (PKA) pathway was not involved in the mediation of gonadotropin action in these cells as gonadotropins did not increase intracellular cAMP formation and inhibition of PKA did not affect gonadotropin-induced phosphorylation of ERK1/2. Activation of ERK1/2 was inhibited by the protein kinase C (PKC) inhibitor GF 109203X as well as by the PKC δ inhibitor rottlerin, and downregulation of PKC δ was inhibited by small interfering RNA (siRNA), highlighting the importance of PKC δ in the gonadotropin signalling cascade. Furthermore, in addition to inhibition by PD98059, gonadotropin-induced ovarian cancer cell migration was also inhibited by verapamil, GF 109203X and rottlerin. Similarly, gonadotropin-induced proliferation was inhibited by PD98059, verapamil, GF 109203X and PKC δ siRNA. Taken together, these results demonstrate that gonadotropins induce both ovarian cancer cell migration and proliferation by activation of ERK1/2 signalling in a calcium- and PKC δ -dependent manner.

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Introduction

Ovarian cancer is the eighth most commonly diagnosed cancer in women and the most lethal of all the gynaecological malignancies, with a 5-year survival around 40% (Jemal *et al.* 2008). Approximately 90% of ovarian cancers are thought to arise from the ovarian surface epithelial (OSE) cell layer or fallopian tube fimbria, but little is known about their aetiology (Dubeau 2008). There is increasing evidence that the hormonal environment surrounding the ovaries

can influence the development of ovarian cancer (Riman *et al.* 2004, Lukanova & Kaaks 2005). One prominent hypothesis is that the gonadotropins FSH and LH enhance ovarian tumourigenesis, which is supported by the fact that ovarian cancer is more common in postmenopausal women in whom serum gonadotropin levels are elevated (Konishi 2006). Additionally, there is a pronounced decrease in the risk of developing epithelial ovarian cancer (EOC) in women using oral contraceptives for more than 10 years, having

multiple pregnancies, or having prolonged lactation, all conditions associated with suppressed gonadotropins in the circulation (Gnagy *et al.* 2000, Modugno *et al.* 2004). Of significance, recent studies of ovarian cyst fluid from malignant and benign ovarian tumours have shown higher levels of FSH and LH in fluid from malignant versus benign tumours (Rzepka-Gorska *et al.* 2004, Thomas *et al.* 2008).

Numerous studies have shown that FSH in particular has a proliferative effect on normal and malignant OSE cell growth (Wimalasena *et al.* 1992, Parrott *et al.* 2001, Syed *et al.* 2001, Ji *et al.* 2004). However, studies have shown conflicting effects of LH on the growth of benign and malignant OSE cells (Zheng *et al.* 2000, Ivarsson *et al.* 2001, Syed *et al.* 2001). Little is known about the role of gonadotropins in other critical events of ovarian tumourigenesis, such as metastasis, although it has been shown that elevated FSH and LH may be important in increasing adhesion of EOC cells (Schiffenbauer *et al.* 2002). Furthermore, gonadotropins have been shown to promote invasion of ovarian cancer cells through the phosphatidylinositol 3-kinase (PI3K) and protein kinase A (PKA) pathways in SKOV-3 ovarian cancer cells (Choi *et al.* 2006). These results suggest that gonadotropins may play an important, yet not fully understood, role in metastatic spread of ovarian cancer.

Mitogen-activated protein kinases (MAPKs) play a pivotal role in transmitting signals from external stimuli such as hormones, stress and chemotherapeutics to cellular responses including proliferation, differentiation and apoptosis (Yoon & Seger 2006). Three subgroups of MAPKs have been identified including extracellular signal-regulated kinases (ERKs), p38 and Jun N-terminal kinases/stress-activated protein kinases. MAPK signalling cascades can be activated by both receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR; Grant *et al.* 2002) and G protein-coupled receptors (GPCRs; Goldsmith & Dhanasekaran 2007). Both FSH and LH signal through their respective GPCRs, FSH receptor (FSHR) and LHR, with the LHR also acting as the receptor for human chorionic gonadotropin (Viswanath *et al.* 2007). Recently, it has been reported that the MAPK signalling cascade is activated by gonadotropins in immortalised OSE cells (Choi *et al.* 2002), leading to upregulation of the EGFR (Choi *et al.* 2005). However, to date, the molecular pathways leading to gonadotropin-induced ERK1/2 activation in EOC have not been described.

The protein kinase C (PKC) family is a group of serine–threonine kinases, with individual isozymes being involved in a variety of cellular functions, including cell growth, memory, survival, apoptosis,

signal transduction, gene expression, migration and hormone action (Dempsey *et al.* 2000, Koivunen *et al.* 2006). PKCs can be subdivided into three major groups based on their structural and functional features including conventional isoforms (cPKC; α , β I, β II and γ) that are diacylglycerol (DAG) sensitive and calcium responsive, novel isoforms (nPKC; δ , ϵ , η and θ) that are DAG sensitive but not calcium responsive, and atypical isoforms (aPKCs; ζ and ι/λ in human/mouse) that are neither DAG nor calcium dependent (Parker & Murray-Rust 2004). The signalling events following activation of PKC are not thoroughly characterised; however, PKC isozymes are known to regulate PI3K, glycogen synthase kinase-3 β and MAPK signalling pathways (Ali *et al.* 2009). PKC α has been specifically implicated in FSH-induced proliferation of the serous subtype EOC cell line HRA (Ohtani *et al.* 2001).

Here, we show that the gonadotropins LH and FSH activate ERK1/2 signalling in EOC cell lines of the serous and clear cell histopathological subtypes leading to both gonadotropin-induced cell migration and proliferation. Furthermore, we demonstrate that both calcium and PKC δ are required for gonadotropin-induced activation of ERK1/2 and consequent migration and proliferation of EOC cells.

Materials and methods

Materials

Human pituitary LH and recombinant FSH were provided by Dr A F Parlow (National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, Torrance, CA, USA). PD98059, a MAPK (MEK) inhibitor; forskolin, an activator of adenylyl cyclase; verapamil hydrochloride, an L-type calcium channel blocker; and dantrolene sodium salt, a ryanodine receptor antagonist, were obtained from Sigma–Aldrich Corporation. The general PKC inhibitor GF 109203X was purchased from Invitrogen. Isobutylmethylxanthine (IBMX), a selective inhibitor of calcium–calmodulin-dependent phosphodiesterase, myristoylated PKA inhibitor amide 14–22 (myrPKAi), PKC δ inhibitor rottlerin and PKC β inhibitor 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione were obtained from Calbiochem (San Diego, CA, USA). Antibodies were purchased from the following companies: phosphorylated Thr202/Tyr204 and total p42/p44 MAPK, Cell Signaling, Beverly, MA, USA; PKC δ , BD Biosciences, North Ryde, NSW, Australia; α -tubulin, Sigma–Aldrich Corporation and GAPDH, Abcam, Cambridge, MA, USA.

Cell culture and treatments

OVCAR-3, a human serous EOC cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA), and OV207, a clear cell adenocarcinoma-derived cell line, was a kind gift from Drs C Conover and K Kalli (Mayo Clinic, Rochester, MN, USA; Conover *et al.* 1998). BSA was obtained at different times from Thermo Trace (Melbourne, VIC, Australia) or from Sigma–Aldrich. All cell lines were cultured in RPMI 1640 (Gibco, Invitrogen) supplemented with 10% FBS (Gibco or SAFC Biosciences, Brooklyn, VIC, Australia) and 0.3 mg/l glutamine (Gibco) at 37 °C in 5% CO₂. Cells were plated at a density of 2 × 10⁵ cells per well in six-well tissue culture plates, and were allowed to attach overnight. After attachment, culture medium was changed to serum-free culture medium containing 0.1% BSA, and after 24 h, cells were treated with 10 nM FSH or LH and agonists or inhibitors as indicated.

SDS-PAGE and western analysis

After treatment, cells were washed twice in ice-cold PBS before the addition of cell lysis buffer (62.5 mmol/l Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, 1 mM sodium pyrophosphate) containing 10% (v/v) protease inhibitor cocktail (Sigma–Aldrich). Cell lysates were sonicated for 2 min (Vibracell, Sonics & Materials, Newton, CT, USA), and were then heated for 5 min at 95 °C. Lysates were electrophoresed on 10% Novex NuPage gels (Invitrogen) at 185 V for 55 min, and were transferred to a nitrocellulose membrane by a wet transfer system (Bio-Rad) at 100 V for 90 min. After blocking with 5% skim milk for 1 h, blots were probed with the primary antibody in 5% BSA overnight at 4 °C before incubation with peroxidase-labelled secondary antibody in 5% skim milk for 1 h at room temperature. Chemiluminescence was detected with Super Signal ECL reagent (Pierce, Rockford, IL, USA) and visualised using the Fujifilm LAS-3000 imaging system (Berthold Australia Pty Ltd, Bundoora, VIC, Australia), and bands were quantitated using Multi Gauge 3.0 (Fujifilm Australia Pty Ltd, Brookvale, NSW, Australia).

Enzyme immunoassay for intracellular cAMP

To measure intracellular cAMP, cells were plated at a density of 5 × 10⁵ cells per well in six-well tissue culture plates, allowed to attach overnight and then serum deprived for 24 h. The cells were preincubated in a serum-free medium containing 100 μM IBMX for

30 min and were treated with FSH, LH or forskolin for 10 min at the doses indicated. Intracellular cAMP levels were measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). Because intracellular cAMP levels were <5 pmol/ml, the samples were acetylated as described by the manufacturer, resulting in a detection limit of 0.1 pmol/ml.

Downregulation of PKCδ by siRNA

Amaxa Nucleofector Technology (Lonza Cologne AG, Cologne, Germany) was used to transfect cells with a commercial PKCδ small interfering RNA (siRNA), target sequence: 5'-CAGCAGCAAGTGCAACAT-3' (Qiagen). Subconfluent cells were harvested and diluted to 1 × 10⁶ cells/ml with Nucleofector solution T. Up to 200 nM of siRNA were added to 100 μl of cell suspension followed by electroporation using program T-016. A non-silencing negative control siRNA, target sequence: 5'-AATTCTCCGAACGTGTCACGT-3' (Qiagen), was used at the same concentration as PKCδ siRNA for all the experiments. RNA was extracted using TRIZOL and reverse transcribed using Superscript III reverse transcriptase according to the manufacturer's instructions (Invitrogen).

Quantitative RT-PCR was performed in triplicate using a TaqMan Gene Expression Assay (*PKCδ*: Hs00178914_m1; Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia). Reagents were aliquoted using the epMotion 5070 automated pipetting system (Eppendorf, Hamburg, Germany). The endogenous reference gene used for normalisation was hydroxymethylbilane synthase (*HMBS*) (Hs00609297_m1), and results are expressed as *PKCδ:HMBS*.

Cell proliferation assay

For proliferation analysis, 1.5–2 × 10⁴ cells were seeded in 48-well plates, and 10 nM FSH, 10 nM LH and/or inhibitors (10 μM PD98059, 25 μM verapamil or 100 nM GF 109230X) were added in 10% FBS-containing medium. After 3 days, media were changed, and fresh gonadotropins and/or inhibitors were added. After 7 days, cells were trypsinised and resuspended in 10% FBS-containing medium, and the cell number was determined using a haemocytometer. Three outside squares were counted for each well, and each condition was analysed in triplicate.

Monolayer wound closure migration assay

For monolayer wounding assays, OV207 cells were plated at a density of 1×10^5 cells per well in 24-well tissue culture plates, and were allowed to attach overnight. Confluent monolayers were scratched using a 10- μ l pipette tip and washed once with serum-free medium. Fresh medium containing 10% FBS and inhibitors (10 μ M PD98059, 25 μ M verapamil, 100 nM GF 109230X or 3 μ M rottlerin) plus or minus 10 nM FSH or LH was added. Additionally, 10 μ M cytosine β -D-arabinofuranoside (Sigma–Aldrich), an inhibitor of DNA synthesis, was included in all wells to exclude any possible proliferation effects. After 22 h, migration was assessed by phase contrast microscopy, documented by digital photography and quantitated using Multi Gauge V3.0 software (Fujifilm). Migration was calculated as the initial wounded (i.e. cell-free) area minus the final cell-free area after migration, and is reported relative to the migration of untreated control cells expressed as 1.0.

Transwell migration assay

Uncoated cell culture inserts (24-well, pore size 8 μ m; BD Biosciences) were seeded with either 2.5×10^4 (OV207) or 1.5×10^5 (OVCAR-3) cells in 100 μ l of medium containing 0.1% FBS and inhibitors. OV207 cells were pretreated with inhibitors (10 μ M PD98059 or 100 nM GF 109230X) for 30 min. OVCAR-3 cells were additionally treated with either 25 μ M verapamil or 3 μ M rottlerin in a similar fashion. Inhibitors did not show any toxicity to the cells over the course of these assays. FSH or LH, 10 nM, was added to the top chamber. Medium containing 10% FBS, inhibitors and 10 μ M cytosine β -D-arabinofuranoside was added to the lower chamber, and it served as a chemotactic agent. After 22 h (OV207) or 48 h (OVCAR-3), non-migrating cells were wiped from the upper side of the membrane with a cotton swab, and cells on the lower side were washed in PBS, fixed in cold methanol (-20°C) for 20 min and air dried. Membranes were excised and mounted on glass slides with ProLong Gold Antifade containing 4',6-diamidino-2-phenylindole, dihydrochloride (Invitrogen) for visualisation of the nuclei. Nuclei were counted using an epifluorescent microscope equipped with a digital camera (Olympus distrene-plasticiser-xylene-71). Each individual experiment had duplicate inserts, and five microscopic fields ($\times 200$ magnification for OV207 and $\times 400$ magnification for OVCAR-3) were counted per insert.

Statistical analysis

Data analysis was performed using SPSS software 16.0 (SPSS Australasia Pty Ltd, Chatswood, NSW, Australia), and results are represented as the mean \pm S.E.M. from at least three independent experiments. Statistical significance was determined by ANOVA with Fisher's least significant difference *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Gonadotropins activate the MAPK/ERK1/2 signalling pathway in EOC cells

OVCAR-3 cells have previously been shown to express the FSHR (Choi *et al.* 2002, Zhang *et al.* 2009). To confirm the expression of both FSHR and LHR, OV207 and OVCAR-3 cells were subjected to immunofluorescent staining (Supplementary Figure 1A and B, see section on supplementary data given at the end of this article), which clearly indicated the presence of both receptors in both cell types. To determine the effects of gonadotropins on ERK1/2 signalling in OV207 and OVCAR-3 EOC cells, cell lines were treated with 10 nM FSH or LH over 5–60 min. Both FSH and LH induced an increase in phosphorylated ERK1/2 in both cell lines with maximal stimulation between 5 and 10 min (Fig. 1A). Pretreatment of cells with PD98059 for 30 min abolished gonadotropin-induced ERK1/2 activation, which was measured after treatment with FSH or LH for 10 min (Fig. 1B and C).

cAMP and PKA are not involved in gonadotropin-induced ERK1/2 activation

Gonadotropins are known to signal through their GPCRs, typically stimulating adenylyl cyclase activity leading to increased cAMP formation and activation of PKA. We therefore determined whether this was the mechanism of gonadotropin-induced ERK1/2 activation in OV207 and OVCAR-3 cells. cAMP levels were determined in OV207 and OVCAR-3 cells after treatment with either 10 nM FSH or LH. No change in cAMP levels was observed after up to 15 min exposure to either gonadotropin (data not shown). To stabilise intracellular cAMP levels, OV207 and OVCAR-3 cells were pretreated with 100 μ M IBMX for 30 min followed by treatment with either 10 or 100 nM FSH or LH for 10 min. Treatment with IBMX alone significantly increased basal intracellular cAMP in OVCAR-3 cells; however, additional treatment with either gonadotropin did not increase cAMP levels

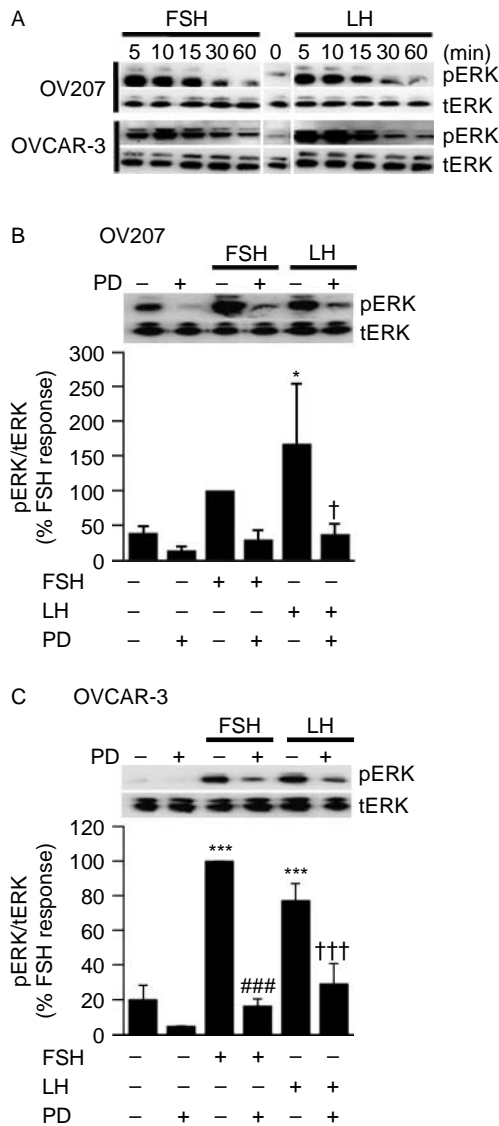


Figure 1 FSH and LH induce ERK1/2 phosphorylation in ovarian cancer cells. (A) OV207 and OVCAR-3 cells were serum deprived for 24 h and treated in a time-dependent manner with 10 nM FSH or LH, and cell lysates were immunoblotted for phosphorylated ERK1/2 (pERK) and total ERK1/2 (tERK). (B) OV207 or (C) OVCAR-3 cells were pretreated with 10 μ M PD98059 (PD) for 30 min, followed by treatment with 10 nM FSH or LH for 10 min, and cell lysates were immunoblotted for pERK1/2 and tERK1/2. Data are expressed as pERK1/2 relative to tERK1/2 (pERK/tERK). All blots are representative of at least three independent experiments. Graphs represent pooled data expressed as mean percentage of FSH response \pm S.E.M. of three individual experiments. * $P < 0.05$ versus untreated control; *** $P < 0.001$ versus untreated control; ### $P < 0.001$ versus FSH alone; † $P < 0.05$ versus LH alone; ††† $P < 0.001$ versus LH alone (by ANOVA).

further (Fig. 2A). cAMP levels were unchanged by either IBMX or gonadotropins in OV207 cells (Fig. 2B). In contrast, increasing concentrations of forskolin led to a dose-dependent increase in intracellular cAMP in both cell lines, with OVCAR-3 cells showing the highest levels of forskolin-induced cAMP (Fig. 2C), confirming that the adenylyl cyclase system was functional in these cell lines. To further exclude the involvement of the PKA pathway in gonadotropin-induced ERK1/2 phosphorylation, cells were pretreated with a myrPKAi, a highly specific inhibitor of PKA, followed by treatment with either FSH or LH for 10 min. Both FSH and LH increased phosphorylation of ERK1/2 in the presence of myrPKAi, confirming that PKA is not involved in gonadotropin-induced ERK1/2 phosphorylation (Fig. 2D).

Calcium plays an important role in gonadotropin-induced ERK1/2 activation

Since cAMP was not involved in gonadotropin-induced ERK1/2 activation in the cell lines analysed, we explored the role of calcium as a possible second messenger in this signalling pathway. Sequestration of extracellular calcium by the chelating agent EGTA significantly reduced gonadotropin-induced phosphorylation of ERK1/2 in OV207 and OVCAR-3 cells, suggesting that calcium influx is required for gonadotropin-induced ERK1/2 activation (Fig. 3A and B). Furthermore, treatment with 25 μ M verapamil for 30 min significantly inhibited gonadotropin-induced ERK1/2 activation (Fig. 3A and B).

We then sought to determine whether intracellular release of calcium plays a role in gonadotropin-induced ERK1/2 activation. Cells were treated with dantrolene, a ryanodine receptor antagonist which blocks the release of calcium from intracellular calcium stores. Dantrolene significantly inhibited gonadotropin-induced ERK1/2 phosphorylation in both cell lines (Fig. 3C and D). Taken together, these results show that gonadotropin-induced ERK1/2 activation is dependent on intracellular calcium, and both intracellular calcium release and calcium influx through L-type voltage-dependent calcium channels need to be active to maintain responsiveness to gonadotropins in EOC cells.

PKC δ mediates gonadotropin-induced ERK1/2 activation

Given the role demonstrated for calcium, but not for PKA, in gonadotropin-induced ERK1/2 activation, we examined a possible role for PKC. Gonadotropin-induced phosphorylation of ERK1/2 was reversed

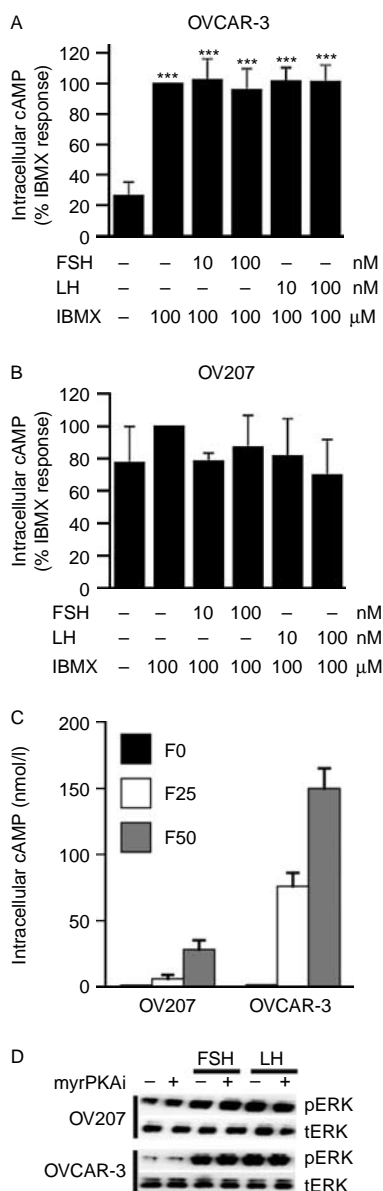


Figure 2 The PKA pathway does not have a role in gonadotropin-induced ERK1/2 phosphorylation in OVCAR-3 and OV207 cells. (A) OVCAR-3 and (B) OV207 cells were serum deprived for 24 h, pretreated with 100 μ M IBMX for 30 min, and then treated with either 10 or 100 nM of FSH or LH for 10 min. Data are the mean \pm s.e.m. of three independent experiments normalised to IBMX treatment alone (OVCAR-3, 3.95 ± 2.95 nmol/l cAMP; OV207, 1.08 ± 0.2 nmol/l cAMP). *** $P < 0.001$ versus untreated control (by ANOVA). (C) OV207 and OVCAR-3 cells were serum deprived for 24 h, and were then treated for 10 min with increasing concentrations of forskolin – 0 μ M (F0), 25 μ M (F25) or 50 μ M (F50). Intracellular cAMP is shown as the mean of two independent experiments \pm s.d. (D) Serum-deprived cells were pretreated with 1 μ M myristoylated PKA inhibitor amide 14–22 (myrPKAi) for 30 min followed by treatment with 10 nM FSH or LH for 10 min. Cell lysates were subjected to immunoblotting for pERK1/2 (pERK) and tERK1/2 (tERK). Blots are representative of three independent experiments.

by the PKC inhibitor GF 109203X (Fig. 4). Since GF 109203X has been shown to efficiently inhibit the PKC isoforms α , β , δ and ϵ at nanomolar concentrations in rat brain (Martiny-Baron *et al.* 1993) and PKC θ in transiently transfected COS-1 cells (Uberall *et al.* 1997), these data support the involvement of one of these PKC isoforms in gonadotropin-induced ERK1/2 activation.

As calcium is involved in gonadotropin-induced activation of ERK1/2, we examined the possible involvement of calcium-dependent PKC isozymes. PKC α and PKC β , but not PKC γ , were detected by western blot analysis in both cell lines (data not shown). siRNA-mediated knockdown of PKC α showed no effects on activation of ERK1/2 in OV207 cells and variable effects on ERK1/2 phosphorylation in OVCAR-3 cells treated with either FSH or LH (Supplementary Figure 2A, see section on supplementary data given at the end of this article and data not shown). Treatment of OVCAR-3 cells with a specific PKC β inhibitor significantly inhibited FSH-induced phosphorylation of ERK1/2 but not LH-stimulated ERK1/2 activation (Supplementary Figure 2B), whereas in OV207 cells, the PKC β inhibitor did not inhibit FSH- or LH-induced phosphorylation of ERK1/2 (Supplementary Figure 2C), and it showed no effect on gonadotropin-induced migration (Supplementary Figure 2D).

To further investigate which PKC isoform mediates gonadotropin-induced ERK1/2 activation, cells were treated with rottlerin, a specific PKC δ inhibitor, at low (3 μ M) concentrations. Treatment with rottlerin reduced gonadotropin-induced ERK1/2 activation in OV207 and OVCAR-3 cell lines (Fig. 5A and B). Similarly, downregulation of PKC δ with siRNA, achieving almost 80% reduction in mRNA up to 72 h (Fig. 5C), significantly inhibited gonadotropin-induced ERK1/2 activation (Fig. 5D and E). Downregulation of PKC δ by specific siRNA did not affect PKC α protein levels (Fig. 5D), with these two PKC isoforms being known to act coordinately, often with opposing effects (Deucher *et al.* 2002).

Gonadotropin-induced migration of EOC cells is dependent on ERK1/2 activation regulated by calcium and PKC δ

Treatment of OV207 and OVCAR-3 cells with 10 nM FSH or LH significantly increased cell migration in comparison to untreated control cells (Fig. 6). PD98059 and GF 109230X significantly inhibited basal and gonadotropin-induced migration in OV207 cells in the monolayer scratch wound

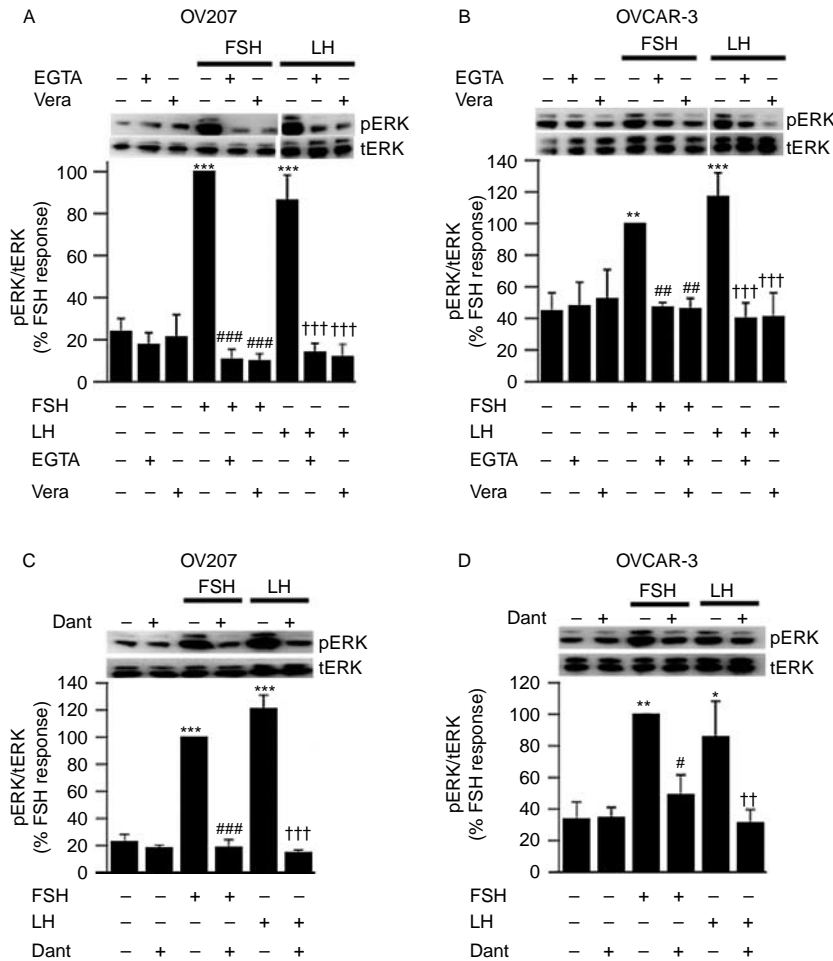


Figure 3 Calcium is required for gonadotropin-induced ERK1/2 phosphorylation. Serum-deprived (A) OV207 or (B) OVCAR-3 cells were pretreated with 3 mM EGTA or 25 μ M verapamil (Vera) for 30 min followed by 10 min of treatment with 10 nM FSH or LH. $^{**}P < 0.01$ versus untreated control; $^{***}P < 0.001$ versus untreated control; $^{\#}P < 0.01$ versus FSH alone; $^{\#\#}P < 0.001$ versus FSH alone; $^{\dagger\dagger\dagger}P < 0.001$ versus LH alone (by ANOVA). (C) Serum-deprived OV207 or (D) OVCAR-3 cells were pretreated with 25 μ M dantrolene (Dant) for 30 min followed by treatment with either 10 nM FSH or LH. $^{*}P < 0.05$ versus untreated control; $^{**}P < 0.01$ versus untreated control; $^{***}P < 0.001$ versus untreated control; $^{\#}P < 0.05$ versus FSH alone; $^{\#\#}P < 0.001$ versus FSH alone; $^{\dagger\dagger}P < 0.01$ versus LH alone; $^{\dagger\dagger\dagger}P < 0.001$ versus LH alone (by ANOVA). All cell lysates were subjected to immunoblotting for pERK1/2 (pERK) and tERK1/2 (tERK), and data are expressed as pERK1/2 relative to tERK1/2 (pERK/tERK). All blots are representative of at least three independent experiments. All graphs represent pooled data expressed as mean percentage of FSH response \pm s.e.m. of three individual experiments.

assay (Fig. 6A and B). In OV207 transwell migration assays, LH-induced migration was significantly inhibited by both PD98059 and GF 109230X, with FSH-induced migration showing a reduced trend in cells treated with these inhibitors (Fig. 6C). Transwell migration assays of OVCAR-3 cells showed inhibition of basal and gonadotropin-induced migration with PD98059 and GF 109230X (Fig. 6D), demonstrating that activated ERK1/2 signalling and PKC signalling are required for gonadotropin-induced cell migration. Gonadotropin-induced cell migration was also inhibited by treatment with verapamil in both cell lines, indicating a significant

role for calcium in this process (Fig. 6B and D). Furthermore, in both cell lines, rottlerin significantly inhibited gonadotropin-induced cell migration (Fig. 6B and D), suggesting that PKC δ -mediated signalling is important in gonadotropin-induced EOC cell migration.

Gonadotropin-induced proliferation of EOC cells is dependent on ERK1/2 activation regulated by calcium and PKC δ

Treatment of OV207 or OVCAR-3 cells with 10 nM FSH or LH significantly increased cell proliferation in comparison to untreated control cells (Fig. 7A and B).

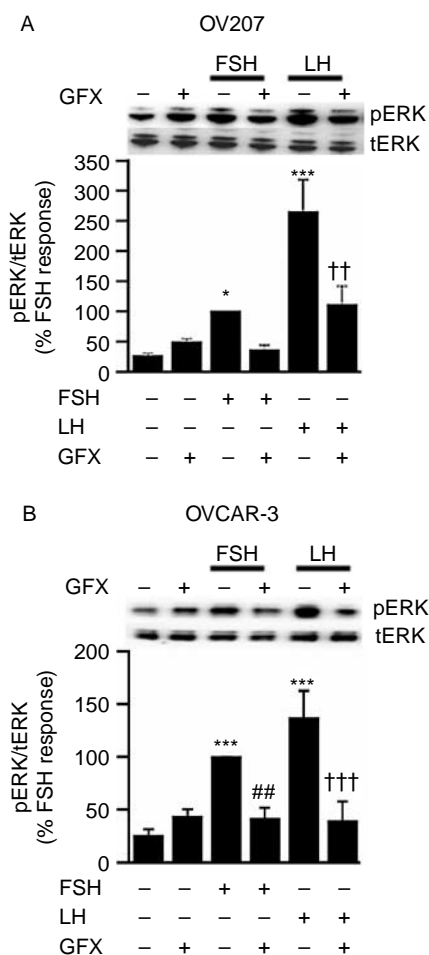


Figure 4 PKC has a role in gonadotropin-induced ERK1/2 phosphorylation. Serum-deprived (A) OV207 or (B) OVCAR-3 cells were pretreated with 100 nM GF 109203X (GFX) for 30 min followed by treatment with 10 nM FSH or LH. Cell lysates were immunoblotted for pERK1/2 and tERK1/2, and data are expressed as pERK/tERK. Data in graphs were pooled and expressed as mean percentage of FSH response \pm S.E.M. of three individual experiments. * $P < 0.05$ versus untreated control; *** $P < 0.001$ versus untreated control; ## $P < 0.01$ versus FSH alone; †† $P < 0.01$ versus LH alone; ††† $P < 0.001$ versus LH alone (by ANOVA).

Proliferation was inhibited by PD98059 (Fig. 7A and B), demonstrating that activated ERK1/2 signalling is required for gonadotropin-induced cell proliferation. Gonadotropin-induced cell proliferation was also inhibited by treatment with verapamil, indicating that calcium influx is required in this process (Fig. 7A and B), and by GF 109203X (Fig. 7A and B), indicating that PKC is involved. Downregulation of PKC δ by siRNA in OVCAR-3 cells reduced both FSH- and LH-induced (Fig. 7C and D) proliferation in OVCAR-3 cells, demonstrating that PKC δ plays an important role in gonadotropin-induced EOC proliferation.

Discussion

Strong epidemiological evidence, largely gathered through studies of postmenopausal women, suggests that elevated levels of serum gonadotropins FSH and LH may have a role in malignant transformation of OSE cells. We have shown that gonadotropin-induced activation of ERK1/2 relies on both calcium- and PKC δ -dependent mechanisms in two EOC cell lines representing the serous and clear cell subtypes. While other *in vitro* studies have shown that gonadotropins induce proliferation in normal and immortalised OSE cells, as well as in EOC cell lines (Wimalasena *et al.* 1992, Parrott *et al.* 2001, Syed *et al.* 2001, Ji *et al.* 2004), little is known about gonadotropin involvement in other aspects of ovarian tumorigenesis such as migration. We have shown that gonadotropin-induced ERK1/2 activation is required for EOC cell migration and proliferation, and that these effects are regulated by both calcium and PKC δ .

The GPCRs for FSH and LH are known to be expressed in both normal OSE cells and EOC cells (Parrott *et al.* 2001, Choi *et al.* 2002, Gebauer *et al.* 2004, Ji *et al.* 2004). Treatment with FSH has been shown to activate the MAPK signalling cascade as indicated by phosphorylation of ERK1/2 in tumourigenic and immortalised normal OSE cells (Choi *et al.* 2002). LH has also been shown to activate ERK1/2 in a non-tumourigenic OSE cell line (Choi *et al.* 2005). However, to date, the molecular pathways leading to gonadotropin-induced ERK1/2 activation in EOC have not been described.

In ovarian granulosa cells, the cells of origin of a small percentage of ovarian tumours, gonadotropins signal by activating the stimulatory G-protein (G_s) of their respective GPCR. The α -subunit of a G_s protein ($G\alpha_s$) stimulates adenylyl cyclase activity causing increased production of cAMP leading to activation of PKA (Hsueh *et al.* 1984, Cotton & Claing 2009). This accepted model of signalling through gonadotropin receptors is not true for FSH and LH in all EOCs, given that both gonadotropins failed to increase intracellular cAMP in the cell lines studied. These findings are consistent with data reported by Choi *et al.* (2002) who demonstrated no increase in basal cAMP after FSH treatment in a non-tumourigenic immortalised cell line (IOSE-29) and its tumourigenic derivative (IOSE-29EC) after up to 60 min incubation, but saw induction of cAMP in human granulosa cells under the same conditions. However, total cAMP levels have been shown to increase up to 1.4-fold in response to incubation with LH for 60 min in the EOC cell line HEY (Slot *et al.* 2006). Additionally, it has been shown

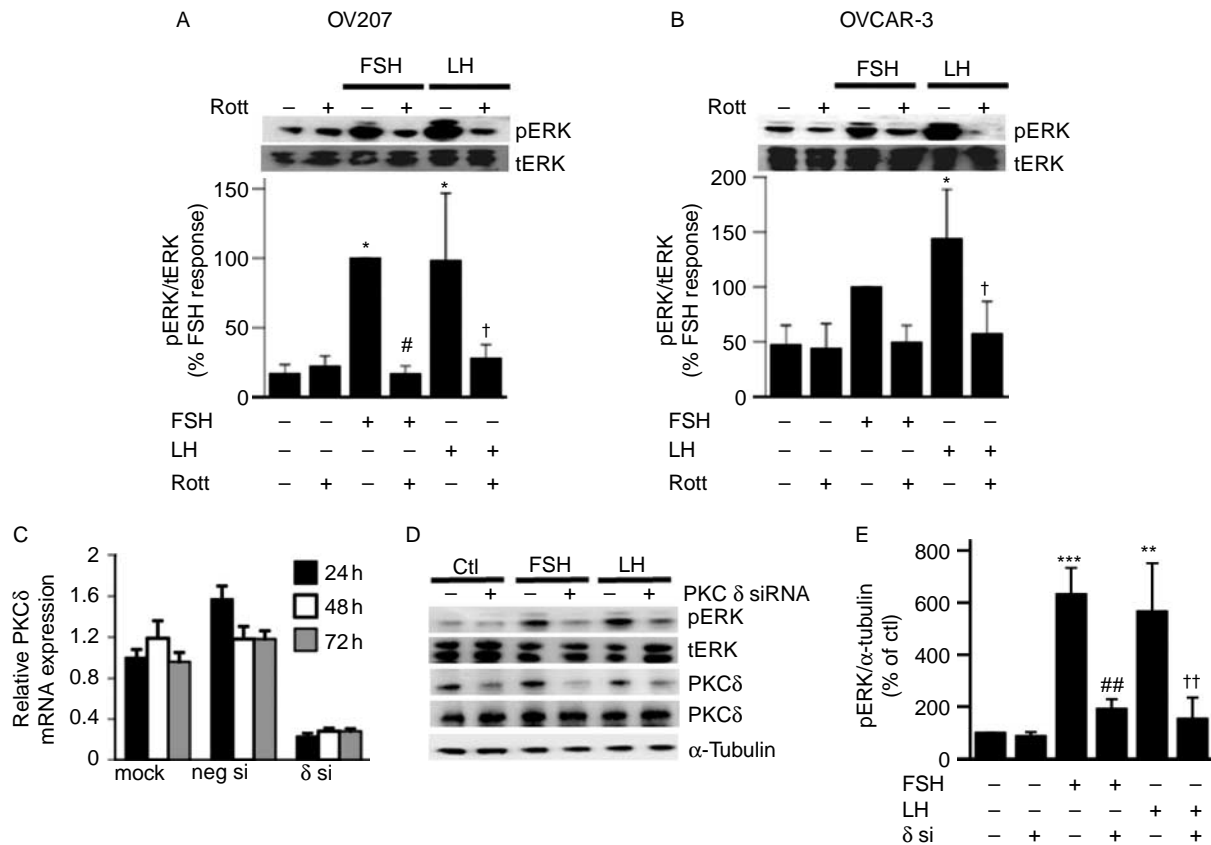


Figure 5 PKC δ mediates gonadotropin-induced ERK1/2 phosphorylation. Serum-deprived (A) OV207 or (B) OVCAR-3 cells were pretreated with 3 μ M rottlerin (Rott) followed by 10 min of treatment with 10 nM FSH or LH. Cell lysates were immunoblotted for pERK1/2 and tERK1/2, and data are expressed as pERK/tERK. Data in graphs were pooled and expressed as mean percentage of FSH response \pm s.e.m. of three individual experiments. * P <0.05 versus untreated ctl; # P <0.05 versus FSH alone; † P <0.05 versus LH alone (by ANOVA). (C) OVCAR-3 cells were transfected in the following manner: no siRNA (mock), non-silencing negative siRNA (neg si) or PKC δ -specific siRNA (δ si). RNA was extracted after 24, 48 and 72 h, and quantitative real-time PCR was performed. Relative levels of PKC δ mRNA normalised to the reference gene *HMBS* and expressed relative to mock transfected cells are shown. (D) OVCAR-3 cells were transfected with either PKC δ siRNA (+) or non-silencing negative siRNA (-). After 48 h, cells were treated with 10 nM FSH or LH, and cell lysates were immunoblotted for pERK1/2, tERK1/2, PKC δ , PKC α and α -tubulin expression. (E) pERK/ α -tubulin ratios were pooled and expressed as mean percentage of untreated control (ctl) cells \pm s.e.m. of three individual experiments performed in duplicate. ** P <0.01 versus untreated ctl; *** P <0.001 versus untreated ctl; ## P <0.01 versus FSH alone; †† P <0.01 versus LH alone (by ANOVA).

that gonadotropins slightly elevate intracellular cAMP levels in a number of immortalised OSE cell lines (IOSE-80, IOSE-120 and IOSE-80PC) at earlier time points of between 5 and 15 min post gonadotropin treatment (Choi *et al.* 2009). Furthermore, inhibiting PKA in normal and malignant OSE cells for 5 days has been shown to reduce gonadotropin-induced cell proliferation (Syed *et al.* 2001). Given that a highly specific inhibitor of PKA did not affect gonadotropin-induced induction of ERK1/2 phosphorylation in the EOC lines in this study, coupled with the fact that gonadotropins did not increase cAMP in these cells, we conclude that gonadotropin-induced ERK1/2 phosphorylation occurs in a cAMP/PKA-independent manner in OVCAR-3 and OV207 cells.

Intracellular calcium plays a pivotal role in cellular function by regulating activities such as cell division and differentiation, and it is a common intermediate between ERK1/2 and GPCRs (Roderick & Cook 2008). A rise in intracellular calcium concentration may occur from two different sources: release of calcium from intracellular stores in the endoplasmic reticulum and/or calcium influx via calcium channels from the extracellular space. Calcium levels are important for signalling and growth in OSE cells, with increased proliferation being seen in response to elevated calcium *in vitro* (McNeil *et al.* 1998, Hobson *et al.* 2000, 2003, Bilderback *et al.* 2002, Wright *et al.* 2002). This effect has been shown to be mediated by ERK1/2 (Hobson *et al.* 2000), PI3K

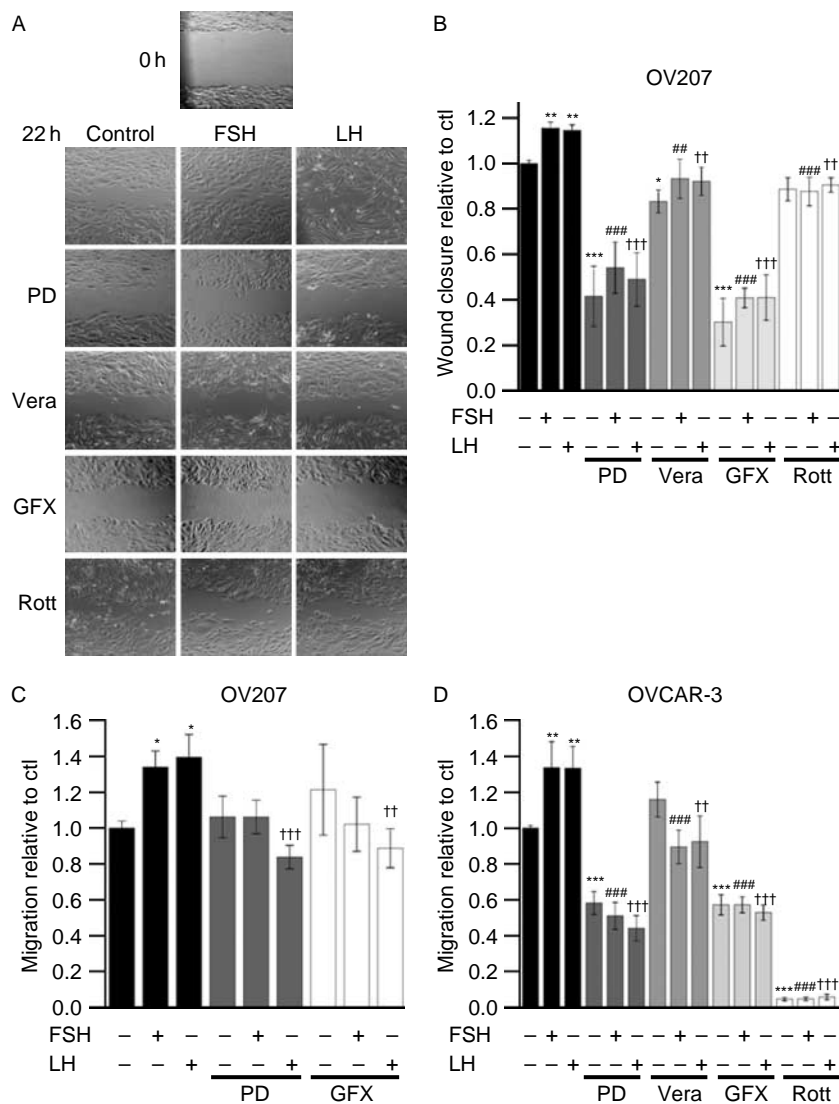


Figure 6 FSH- and LH-induced migration requires activation of MAPK signalling and is regulated by calcium and PKC δ . (A) Confluent OV207 cells were wounded, and then treated with 10 nM FSH or LH in the presence of either PD98059 (PD, 10 μ M), verapamil (Vera, 25 μ M), GF 109203X (GFX, 100 nM) or rottlerin (Rott, 3 μ M) for 22 h. Representative photomicrographs are shown at 0 and 22 h post treatment. (B) Scratch wound closure is expressed as closure relative to that of untreated control (ctl) cells for all experiments. Data are pooled mean \pm S.E.M. from three individual experiments performed in duplicate. (C) OV207 and (D) OVCAR-3 cells were seeded into transwell chambers and treated with either 10 nM FSH or LH in the presence of PD98059 (PD, 10 μ M), GF 109203X (GFX, 100 nM), verapamil (Vera, 25 μ M) or rottlerin (Rott, 3 μ M). Following incubation for 22–48 h, migration was assessed by staining and counting the nuclei of the cells on the underside of the membrane. Data are pooled mean \pm S.E.M. from three individual experiments performed in duplicate. * P <0.05 versus untreated ctl; ** P <0.01 versus untreated ctl; *** P <0.001 versus untreated ctl; ### P <0.01 versus FSH alone; ### P <0.001 versus FSH alone; †† P <0.01 versus LH alone; ††† P <0.001 versus LH alone (by ANOVA).

(Bilderback *et al.* 2002) and the calcium-sensing receptor CaR (Hobson *et al.* 2003). We have shown that FSH- and LH-induced ERK1/2 activation in EOC cell lines is dependent on both extra- and intracellular calcium. Furthermore, our data suggest that gonadotropin-induced ERK1/2 activation is mediated by calcium influx through L-type voltage-dependent calcium channels. The mechanism by which

gonadotropins evoke calcium influx through these channels is unknown. Touyz *et al.* (2000) have found that the growth factor type I receptor FSHR, a splicing variant of the FSHR, is able to evoke calcium influx in response to its ligand FSH via L-type voltage-dependent calcium channels in transfected HEK293 cells. Additionally, granulosa cells transfected with the ovine growth factor type I receptor FSHR showed

cAMP-independent activation of the ERK1/2 signalling cascade upon FSH stimulation, which could be abolished by treatment with the intracellular calcium chelator BAPTA/AM or EGTA (Babu *et al.* 2000), demonstrating dependence on extra- and

intracellular calcium for activation of ERK1/2 signalling. Our data also support calcium-dependent but cAMP/PKA-independent gonadotropin-induced activation of ERK1/2 signalling in EOC.

We have shown that calcium is a key regulator of gonadotropin-induced EOC cell migration and proliferation via activation of ERK1/2. In particular, verapamil decreased FSH- and LH-induced migration in OV207 cells and proliferation in both cell lines. Verapamil has also been shown to reduce cancer cell migration in murine mammary carcinoma and human melanoma cell lines (Yohem *et al.* 1991, Todaro *et al.* 2003). In a phase II trial, the cytostatic inhibitor of calcium influx carboxamidotriazole has been shown to stabilise some patients with EOC who had relapsed for between 6 and 13 months (Hussain *et al.* 2003). Our data also support inhibition of calcium influx as a possible therapeutic approach to combatting EOC progression.

There is increasing evidence that PKC is involved in gonadotropin-induced signalling in granulosa cells in a cAMP/PKA-independent manner (Pennybacker & Herman 1991, Babu *et al.* 2000). In the present study, the PKC inhibitor GF 109203X significantly inhibited gonadotropin-induced ERK1/2 phosphorylation, migration and proliferation, demonstrating that EOCs are dependent on PKC signalling in a number of cellular processes. Using both pharmacological inhibition of PKC δ with rottlerin and the more targeted approach of gene-specific siRNA downregulation, we have shown that the calcium-independent PKC δ isoform is important for gonadotropin-induced ERK1/2 phosphorylation and proliferation. Rottlerin has been widely used and accepted as an inhibitor of PKC δ , although its specificity has been questioned. It is significant that we were able to confirm the involvement of PKC δ using siRNA-mediated knock-down. Ohtani *et al.* (2001) have shown by broad pharmacological inhibition that PKC is involved in

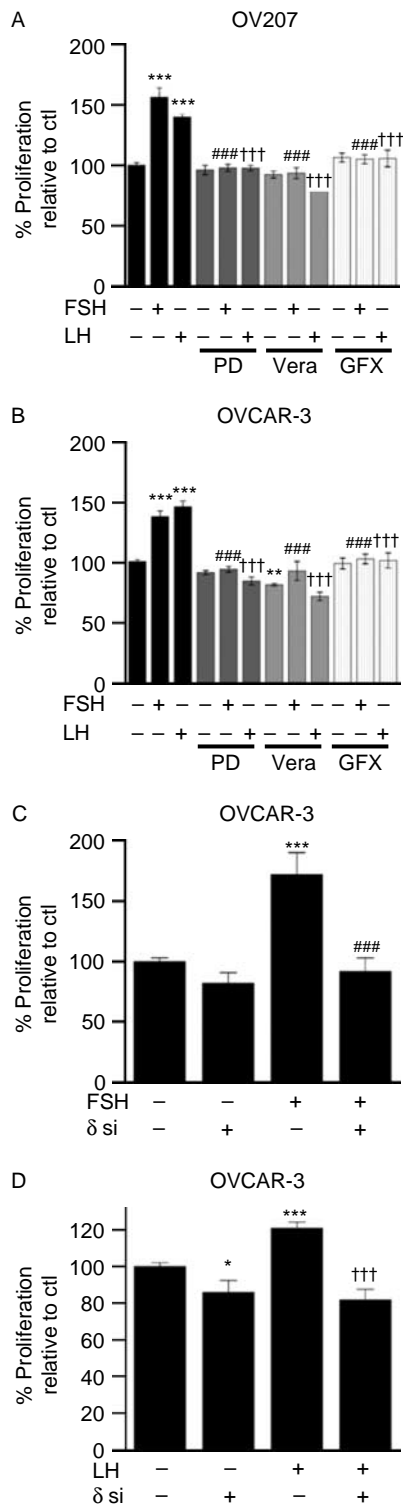


Figure 7 FSH- and LH-induced proliferation requires activation of MAPK signalling and is regulated by calcium and PKC δ . (A) OV207 and (B) OVCAR-3 cells were treated with 10 nM FSH or LH in the presence of either PD98059 (PD, 10 μ M), verapamil (Vera, 25 μ M) or GF 109203X (GFX, 100 nM) for 7 days, and proliferation was assessed by counting the cell number. (C) OVCAR-3 cells were transfected with either non-silencing negative siRNA or PKC δ -specific siRNA, and were treated with 10 nM FSH or LH. Proliferation was assessed after 7 days by direct cell counts. Cell proliferation is expressed as percentage relative to that of untreated control (ctl). Data are pooled mean \pm s.e.m. from three to five individual experiments performed in triplicate. ** P <0.01 versus untreated ctl; *** P <0.001 versus untreated ctl; ### P <0.001 versus FSH alone; ††† P <0.001 versus LH alone (by ANOVA).

the growth stimulatory action of FSH on EOC cells and that FSH upregulates PKC α , suggesting that this isoform may play a role in the proliferative effects of FSH. At least in OV207 cells, downregulation of PKC α with siRNA did not inhibit ERK phosphorylation. We have shown here that PKC δ plays an important role in FSH- and LH-induced migration and proliferation of EOC.

PKC δ has roles in a range of cell functions such as proliferation (as both a positive and negative regulator), differentiation, apoptosis (as both a positive and negative regulator) and tumour suppression (Jackson & Foster 2004, Chen & Chen 2009). In MCF-7 breast cancer cells, oestrogen-induced ERK1/2 activation and subsequent proliferation were blocked by inhibition of PKC δ , demonstrating that PKC δ has the ability to activate the ERK1/2 cascade in another hormone-dependent cancer (Keshamouni et al. 2002). There is also evidence that PKC δ can enhance cell motility, possibly by affecting the expression and activity of β 1 integrins and focal adhesion kinase (Chen et al. 2007, Brenner et al. 2008). Furthermore, PKC δ has been shown to act to promote invasiveness in prostate and breast cancer cells (Kiley et al. 1999, Kharait et al. 2006, Villar et al. 2007), and rottlerin has been shown to completely prevent invasion induced by the integrin-activating peptide PHSRN in DU-125 prostate cancer cells (Zeng et al. 2006). We have shown that both FSH and LH induce migration in ovarian cancer cells that is dependent upon ERK1/2 and PKC δ signalling. In apparent contrast to our findings, Choi et al. (2006) have recently reported gonadotropin-induced invasion in SKOV-3 cells that was dependent on activation of matrix metalloproteases through PKA and PI3K pathways. It is possible that ERK1/2 and PKC δ might be critical for gonadotropin-induced cell motility in EOC cells, but may not be involved in proteolysis-dependent invasion.

The importance of both calcium and PKC δ in the activation of ERK1/2 has recently been described in cardiac fibroblasts. In these cells, angiotensin II-induced ERK1/2 activation has been shown to rely on both calcium- and PKC δ -dependent mechanisms (Olson et al. 2008). Furthermore, angiotensin-II-induced activation of Janus kinase 2 has also been shown to be dependent on PKC δ and calcium involving Pyk2, a non-receptor tyrosine kinase which has been described as a convergence point between GPCRs and activation of the MAPK signalling pathway (Della Rocca et al. 1999, Frank et al. 2002). In HEK293 cells transfected with GnRH receptor, PKC δ and Pyk2 mediate GnRH-induced ERK1/2

activation (Farshori et al. 2003), indicating that PKC δ and Pyk2 may work cooperatively to activate ERK1/2 upon hormone-induced signalling.

The results of the present study suggest that both FSH and LH increase the migration and proliferation of EOC cells through activation of the MAPK/ERK1/2 pathway in a PKC δ - and calcium-dependent manner. Targetting of these pathways and second messengers may offer new therapeutic options for the treatment of EOC.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-09-0152>.

Declaration of interest

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

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