Activation of the prostaglandin FP receptor in human granulosa cells

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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has regulatory (mainly luteolytic) effects in the ovary but the mechanism of action is not completely understood. Reverse transcriptase–polymerase chain reaction (RT–PCR) techniques were used to demonstrate the presence of mRNA encoding the $PGF_{2\alpha}$ receptor (FP receptor) in human granulosa–lutein cells. Specific primers for the amplification of cDNA were designed and yielded a single product of 696 bp corresponding to the FP receptor. The identity of this product was verified by sequencing. Fluprostenol, a selective FP receptor agonist, activated phospholipase C (PLC) and increased intracellular free calcium concentration, confirming the functional activation of the receptor. We have demonstrated by Western blotting that granulosa cells express PLC- β and PLC- γ isoforms. The cells responded to pervanadate with increased PLC activity and increased tyrosine phosphorylation, demonstrating a functional PLC- γ tyrosine kinase pathway. However, fluprostenol did not provoke any detectable tyrosine phosphorylation. Moreover, the effect of fluprostenol was inhibited through protein kinase C stimulation by phorbol 12,13-dibutyrate, and was not affected when cells were treated with phenylarsine oxide, which blocks tyrosine phosphorylation. These results suggest that the FP receptor activates PLC- β rather than PLC- γ isoforms. Fluprostenol-induced activation was pertussis toxin resistant. Granulosa cells express G proteins of the G_q family (resistant to pertussis toxin) and mRNA for both $G\alpha_q$ and $G\alpha_{11}$ has been identified by RT–PCR. In conclusion, human granulosa cells have a functional FP receptor the effects of which are mediated through PLC- β activation probably via $G_{q/11}$.

Introduction

Prostaglandins (PGs) are involved in the regulation of granulosa and luteal cell function but their mechanism of action is not completely understood. PGE_2 has luteotrophic effects both *in vivo* and *in vitro* (McNatty *et al.*, 1975) and stimulates progesterone output by human granulosa cells obtained from follicles at different stages of development (McNatty *et al.*, 1975). The effect of PGE_2 is associated with an increase in cyclic adenosine-3',5'-monophosphate (cAMP) and is mediated by prostaglandin EP_2 receptors coupled to adenylyl cyclase (Dennefors *et al.*, 1982; Michael *et al.*, 1993; López Bernal *et al.*, 1995). By contrast, the action of $PGF_{2\alpha}$ appears to be more complex. In cultured human granulosa cells $PGF_{2\alpha}$ inhibits progesterone output and antagonises the stimulatory effect of hCG (McNatty *et al.*, 1975; Michael and Webley, 1991); a similar luteolytic effect has been described in several primate and non-primate species (Thomas *et al.*, 1978; Hamberger *et al.*, 1979; Pate and Condon, 1984). The antagonodotrophic action of $PGF_{2\alpha}$ may involve inhibitory effects at sites both before and after adenylyl cyclase activation, probably mediated by protein kinase C (PKC) activation (Michael and Webley, 1993) but it could also result from potentiation of the inhibitory effects of

other luteolytic agents, for example endothelin-1 (Prins *et al.*, 1994; Liebermann *et al.*, 1996). The presence of $PGF_{2\alpha}$ receptors (FP receptors) in human and non-primate granulosa–luteal cells has been demonstrated by binding studies (Powell *et al.*, 1974) and the FP receptor has been cloned from ovarian complementary DNA (cDNA) libraries in mice, rats and sheep (Lake *et al.*, 1994; Kitanaka *et al.*, 1994; Sakamoto *et al.*, 1994; Sugimoto *et al.*, 1994; Graves *et al.*, 1995). Ristimäki *et al.* (1997) have cloned a partial cDNA sequence of the FP receptor in human granulosa cells. The expression of mRNA encoding FP receptor was increased by hCG administration *in vitro* (Ristimäki *et al.*, 1997).

FP receptors belong to the G protein-coupled receptor superfamily and they are thought to operate through activation of the inositol phospholipid pathway (Houmard *et al.*, 1992). The hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phospholipase C (PLC) generates two molecules with second messenger function: inositol 1,4,5-trisphosphate which mobilizes calcium from the sarcoplasmic reticulum and 1,2-diacylglycerol which activates PKC. Houmard *et al.* (1992) reported that $PGF_{2\alpha}$ is a potent activator of PLC in a primate (rhesus monkeys) corpus luteum. Moreover, $PGF_{2\alpha}$ increases intracellular free calcium ($[Ca^{2+}]_i$) in human granulosa cells (Currie *et al.*, 1992). However, uncertainty remains as to whether PLC activation and changes in $[Ca^{2+}]_i$ are required for $PGF_{2\alpha}$ to exert its luteolytic effect. For example, in marmoset

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monkeys in the late luteal phase $\text{PGF}_{2\alpha}$ has a strong luteolytic and antigonadotrophic effect which apparently occurs without PLC activation (Michael and Webley, 1993). Moreover, phosphatidylinositol hydrolysis can be catalysed not only by G protein-dependent PLC isoenzymes (PLC- β family), but also by a variety of receptor-tyrosine kinase-dependent enzymes (PLC- γ family). It has been reported that $\text{PGF}_{2\alpha}$ rapidly increases tyrosine phosphorylation in some cell lines (Watanabe *et al.*, 1994) but is not known whether this happens in granulosa cells.

The purpose of this paper was to investigate the second messenger pathway for FP receptors in cultured human granulosa cells. Fluprostenol, a synthetic $\text{PGF}_{2\alpha}$ analogue, which is a selective FP receptor agonist (Dukes *et al.*, 1974; Carrasco *et al.*, 1996) was used and its effects on PLC- β and PLC- γ activation were studied. The presence of mRNA encoding the FP receptor was confirmed and mRNAs encoding two distinct G proteins, $\text{G}\alpha_q$ and $\text{G}\alpha_{11}$, which are known to activate PLC- β , were demonstrated.

Materials and Methods

Materials

Materials used in this study were purchased from the following sources: [^3H]myo-inositol ($1.5 \text{ TBq mmol}^{-1}$), horseradish-peroxidase-conjugated sheep anti-mouse IgG, horseradish-peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence detection kits (ECL) were obtained from Amersham International plc (Amersham). Oxytocin was from Calbiochem-Novabiochem (Nottingham). $\text{PGF}_{2\alpha}$, sodium orthovanadate, phorbol 12,13-dibutyrate (PDBu), phenylarsine oxide (PAO), pertussis toxin, thapsigargin and fatty acid-free bovine serum albumin (BSA) were from Sigma Chemical Co. Ltd (Poole). Anti-phospholipase C antibodies G12 (PLC- $\beta 1$), Q-15 (PLC- $\beta 2$), C-20 (PLC- $\beta 3$), C-18 (PLC- $\beta 4$), 1249 (PLC- $\gamma 1$) and C-19 (PLC- $\gamma 2$) and their respective cognate peptides were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine mouse monoclonal antibody 4G-10 was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Fura-2-acetoxymethyl ester and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Tissue culture reagents were from Life Technologies (Paisley). All other reagents were commercial preparations of the highest available purity. Fluprostenol was kindly supplied by R. Coleman (formerly at Glaxo, UK).

Preparation and incubation of human granulosa cells

Granulosa cells were collected with consent of the patients and approval by the Central Oxfordshire Ethics Committee from women undergoing routine oocyte retrieval for *in vitro* fertilization. Follicular growth was stimulated by daily injection of gonadotrophins in patients previously desensitized with a long course of GnRH (López Bernal *et al.*, 1995). Follicles were aspirated 35 h after administration of 10 000 iu of hCG (Profasi, Serono Labs UK, Ltd, Hertfordshire). After removal of any oocytes present, follicular fluids were centrifuged at 340 g at room temperature for 10 min through Ficoll-Paque

(Pharmacia, St Albans) to remove blood cells. Granulosa cell layers were subsequently dispersed with collagenase (Sigma Co. Ltd, Poole; diluted to 300 U ml^{-1}) and Trasylol (Bayer UK, Newbury; diluted to 100 U ml^{-1}) for 20 min at 37°C in $1 \text{ mmol CaCl}_2 \text{ l}^{-1}$. Viability of cells after dispersion was 80% as detected by Trypan blue staining and the yield per patient was $0.4\text{--}1.5 \times 10^6$ cells.

Cells were seeded at $40 \times 10^3 \text{ cells cm}^{-2}$ in fibronectin-coated flasks and cultured in Ham's F12:DEM (1:1 by volume) medium containing 10% (v/v) fetal calf serum, 10% (v/v) horse serum, $1 \text{ mmol vitamin E l}^{-1}$, $20 \text{ nmol selenium l}^{-1}$ and $100 \text{ U penicillin/streptomycin ml}^{-1}$. Under these conditions the cells retained 3β -hydroxysteroid dehydrogenase and aromatase activities and remained responsive to hCG by increasing progesterone output (López Bernal *et al.*, 1995). The cells were used within the first week in culture.

[^3H]inositol labelling

Labelling of phosphoinositides was performed by incubating granulosa cells with $0.25 \text{ MBq } [^3\text{H}]\text{myo-inositol ml}^{-1}$ in Waymouth culture medium complemented with penicillin, streptomycin and 2% (v/v) FCS for 48 h (Carrasco *et al.*, 1996). The cells were stimulated with fluprostenol for 30 min or pervanadate for 15 min in a Hank's balanced salt solution (HBSS) buffer containing $1 \text{ mg fatty acid-free BSA ml}^{-1}$ and $12 \text{ mmol LiCl l}^{-1}$ in a final volume of $250 \mu\text{l}$. The reaction was stopped with chloroform:methanol:hydrochloric acid (50:100:1, v:v:v). Inositol phosphates (IPs) in the water-soluble phase were separated and quantified according to the method of Bone *et al.* (1984) using Dowex resin columns. Aliquots of the lipid phase were counted for the determination of the labelling of the phospholipids.

Measurements of free $[\text{Ca}^{2+}]_i$

Measurements of $[\text{Ca}^{2+}]_i$ were carried out in granulosa cells grown on coverslips. The coverslips were loaded with $5 \mu\text{mol Fura-2 acetoxymethyl ester l}^{-1}$ in HBSS in the presence of 0.1% (w/v) Pluronic F-127 detergent for 30 min at 37°C in the dark. The cells were washed with HBSS and stored at room temperature until fluorometry. Fluorometric measurements were made by the method of Grynkiewicz *et al.* (1985) in a Shimadzu RF 5001 spectrofluorimeter (Howe, Bicster) controlled by a Viglen 386 SX PC, equipped with a dual-wavelength determination software (Shimadzu), as described by Asbóth *et al.* (1996). Free intracellular calcium was calculated from the ratio of fluorescence emitted at 510 nm using excitation wavelength of 350 and 380 nm. The depletion of intracellular Ca^{2+} storages was undertaken by 10 min pretreatment with $100 \text{ nmol thapsigargin l}^{-1}$. Experiments under low extracellular Ca^{2+} conditions were carried out in HBSS without Ca^{2+} and Mg^{2+} in the presence of $100 \mu\text{mol ethyleneglycol-bis-(}\beta\text{-aminoethyl ether)-N,N,N',N'-tetraacetic acid l}^{-1}$ (Carrasco *et al.*, 1996).

Immunodetection of phospholipase C

PLC isoforms were detected by western blotting in membrane and cytosol fractions of granulosa cells essentially as

described by Phaneuf *et al.* (1996). Proteins were separated by SDS-PAGE (7.5% acrylamide) for 45 min at 200 V, and transferred to nitrocellulose using a Bio-Rad semi-dry electroblotting apparatus with 0.025 mol Tris l^{-1} , 0.2 mol glycine l^{-1} , 20% methanol, pH 8.6 at 13 V for 2 h. After transfer, the nitrocellulose papers were incubated in PBS-Tween (phosphate-buffered saline, Tween-20 0.1%, v/v), containing 2% skimmed milk overnight at room temperature. Nitrocellulose papers were then incubated with anti-phospholipase C antibodies at a 1:1000 dilution for 1 h at 4°C in PBS-Tween containing 0.15% skimmed milk. The blots were washed three times in PBS-Tween, and then incubated with goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase at a 1:1000 dilution for 1 h at room temperature in PBS-Tween. Finally the blots were washed three times in PBS. Chemiluminescence detection (ECL) was carried out according to the technical notes provided by Amersham, with Kodak X-Omat XAR film.

Tyrosine phosphorylation assay

Treatments with fluprostenol, pervanadate or oxytocin were carried out on granulosa cells grown on 25 cm² plastic culture flasks as described by Carrasco *et al.* (1996). Briefly, cells were washed with HBSS, then incubated with 1 µmol fluprostenol or oxytocin l^{-1} for 30 min at 37°C, or with pervanadate (400 µmol sodium orthovanadate l^{-1} , 2 mmol H₂O₂ l^{-1}) for 15 min at 37°C. The reactions were stopped by removing the medium and by washing with ice-cold HBSS. The cells were scraped from the flasks and pelleted by centrifugation at 1000 g for 15 min. The pellets were solubilised in SDS buffer containing 8 mol urea l^{-1} and heated at 95°C for 10 min before electrophoresis.

After transfer, the nitrocellulose was saturated with 10% BSA in PBS-Tween for 30 min at 4°C. Nitrocellulose papers were then incubated with anti-phosphotyrosine mouse monoclonal antibodies at 1:1000 overnight at 4°C in the same solution. The blots were washed three times in PBS-Tween, and then incubated with mouse antibodies coupled to horseradish peroxidase at 1:10 000 for 1 h at room temperature in PBS-Tween containing 1.5% BSA. Finally the blots were washed three times in PBS-Tween. Antibody complexes were detected by ECL using Kodak X-Omat XAR film.

RNA isolation

Total cellular RNA was prepared using a modification of the guanidine isothiocyanate method (Chirgwin *et al.*, 1979). Briefly, cells (approximately 10⁶) were solubilized with guanidium isothiocyanate and layered onto a 8 ml cushion of caesium chloride and sedimented at 175 000 g for 20 h at 20°C, in a Beckmann SW 41Ti-rotor. RNA concentrations were determined by UV absorption at 260 nm. The 28/18S ribosomal RNA integrity was assessed in agarose gel electrophoresis.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Synthesis of cDNA was carried out with the Reverse-Transcript kit (R&D Systems Europe Ltd, Abingdon). Briefly,

2 µg of RNA were annealed to oligo d(T) primers. The cDNA was used immediately for PCR or stored at -20°C until use.

The selection of the polymerase chain reaction (PCR) primers was performed using the Wisconsin Package (Genetics Computer Group). On the basis of the human sequence for FP receptor (Abramovitz *et al.*, 1994), Gα_q protein (Chen *et al.*, 1996) and Gα₁₁ protein (Jiang *et al.*, 1991) the following oligonucleotides were chosen:

Forward and reverse primers for FP receptor were 1006 5' GTCTCCTGTATTTGTTGGAGCC and 1701 5' GAG CCGTGATCTCAAAATAACC, respectively. These primers would amplify a 696 bp fragment.

Forward and reverse primers for Gα_q were 357 5' GGA CAGGAGAGAGTGGCAAG and 864 5' TTTCTTCTCTCT GACCTTTGGC, respectively. These primers would amplify a 508 bp fragment.

Forward and reverse primers for Gα₁₁ were 383 5' GCT ACTCGGAGGAGGACAAG and 911 5' GACTCCACCAGG ACTTGGTC, respectively. These primers would amplify a 529 bp fragment.

PCR was performed in a final volume of 10 µl containing 100 ng cDNA, 10 mmol Tris-HCl l^{-1} , pH 8.3, 50 mmol KCl l^{-1} , 1.5 mmol MgCl₂ l^{-1} , 0.001% (w/v) gelatin, 200 µmol of each deoxynucleotide triphosphate (dNTP) l^{-1} , 1 µmol of each primer l^{-1} and 0.25 U AmpliTaq DNA polymerase. The PCR reaction was: 94°C for 5 min, then 30 cycles of 94°C for 1 min, 60°C (for FP receptor) or 62°C (for Gα_q protein) or 59°C (for Gα₁₁ protein) for 1 min and 72°C for 2 min, with a final elongation step of 72°C for 5 min. The products generated were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized by UV irradiation. The identity of the fragments was verified by sequencing with thermo sequenase radiolabelled terminator cycle sequencing kit (Amersham International, Aylesbury).

Statistical analysis

Estimations were carried out with a minimum of four replicates and experiments were performed at least twice using granulosa cells obtained from different donors. Statistical comparisons were performed using Student's *t* test. Differences were considered significant if *P* < 0.05.

Results

Expression of FP receptor gene in human granulosa cells

The presence of FP receptor mRNA in human granulosa cells was demonstrated by RT-PCR analysis using FP receptor-specific primers that were designed to produce a 696 bp fragment. This resulted in the amplification of a single band for the three patients tested (Fig. 1). The identity of the PCR product was confirmed by sequencing which showed 100% homology with the published sequence for the FP receptor (Abramovitz *et al.*, 1994).

Effects of fluprostenol on the formation of [³H]IPs and intracellular calcium concentration

Fluprostenol induced a concentration-dependent increase in total [³H]IPs, with an EC₅₀ value of 26 nmol l^{-1} and a maximal

Fig. 1. Expression of FP receptor gene in human granulosa cells. RT-PCR analysis of FP receptor produced the expected 696 bp band (lanes 2, 3 and 5; three different donors). A PCR sample without RNA transcribed product was loaded in lane 4 (blank). Lanes 1 and 6 contained DNA markers, the molecular size (bp) of which is given on the left. The identity of the PCR products was confirmed by sequencing.

response at $1 \mu\text{mol l}^{-1}$ (Fig. 2a). Moreover fluprostenol increased $[\text{Ca}^{2+}]_i$ with an EC_{50} value of 12 nmol l^{-1} (Fig. 2b). These results demonstrate that human granulosa cells possess a functional FP receptor with high affinity for fluprostenol. This receptor stimulates PLC and increases $[\text{Ca}^{2+}]_i$, probably by Ca^{2+} mobilization from the endoplasmic reticulum, as supported by the fact that depletion of intracellular Ca^{2+} stores with thapsigargin (Thastrup *et al.*, 1990) inhibited the rise in $[\text{Ca}^{2+}]_i$ induced by fluprostenol (Fig. 2b insert). Moreover, high extracellular Ca^{2+} was required for fluprostenol to have a full stimulatory effect (Fig. 2b insert).

Identification of phospholipase C isoforms

The presence of phospholipase C isoforms in human granulosa cells is demonstrated by Western blotting (Fig. 3a, b). PLC- β 1 migrated as two immunoreactive bands at 154 and 100 kDa. Preabsorbing anti-PLC- β 1 with its cognate peptide resulted in the loss of both bands (Fig. 3a). This indicates that the 100 kDa band is immunologically related to the main PLC- β 1. This isoform is known to be highly sensitive to proteolysis, even when cell extracts are freshly prepared in the presence of protease inhibitors (Suh *et al.*, 1988; Park *et al.*, 1993). PLC- β 2 and PLC- β 3 isoforms migrated at a molecular mass of approximately 140 and 155 kDa, respectively. Both bands were lost when the antibodies were first preincubated with their cognate peptides. The antibody raised against PLC- β 4 failed to detect this isoform (Fig. 3b).

A strong band of 148 kDa corresponding to PLC- γ 1 and a smaller band at 90 kDa which is immunologically related to PLC- γ 2 were also detected (Fig. 3a, b). All the PLC isoforms were found mainly in the cytosolic fraction, although PLC- β 1 and PLC- γ 1 appeared to be slightly associated with the membrane fraction.

Involvement of a PLC- β pathway

The presence of PLC- β isoforms in myometrial cells is in agreement with their involvement in G protein-mediated

Fig. 2. Concentration-response curve of fluprostenol-stimulated (a) formation of $[\text{^3H}]\text{IPs}$ and (b) $[\text{Ca}^{2+}]_i$ mobilization in cultured human granulosa cells. (a) Cells were labelled with $[\text{^3H}]\text{myo-inositol}$ and challenged with fluprostenol in the presence of LiCl. (b) Granulosa cells grown on coverslips were loaded with fura-2/acetoxymethyl ester and stimulated with fluprostenol. The inset shows the effect of removing extracellular Ca^{2+} or depleting intracellular Ca^{2+} stores with thapsigargin on the Ca^{2+} peaks provoked by $100 \text{ nmol fluprostenol l}^{-1}$. Each point represents the mean $\pm$ SD of quadruplicate determinations from a single experiment. Similar results were obtained with cells from a different donor.

Fig. 3. Identification of phospholipase C (PLC) isoforms in human granulosa cells. Proteins were resolved by polyacrylamide gel electrophoresis and PLC isoforms were revealed by western blotting and enhanced chemiluminescence (ECL) detection. The positions of the molecular mass standards (kDa) are indicated on the left. (a) Antibodies were preincubated with (+) and without (-) their respective cognate peptides before immunodetection. (b) Cellular distribution of PLC isoforms in membrane (M) and cytosolic (C) fractions. Results are from a representative experiment, and similar findings were obtained with cells from another donor.

receptor signalling. By contrast, PLC- γ isoforms are regulated by tyrosine kinase activity (Blake *et al.*, 1993). Pervanadate, an inhibitor of tyrosine phosphatases (Gordon, 1991), was used to study PLC- γ activation in human granulosa cells and to compare the response to that of fluprostenol. Pervanadate provoked a high level of tyrosine phosphorylation as demonstrated by immunoblotting with anti-phosphotyrosine antibody (Fig. 4). The identity of the phosphorylated proteins was not determined, but they may represent proteins with molecular mass similar to those of PLC- γ isoforms (for example 148 and 90 kDa). Neither fluprostenol nor oxytocin, the receptors of which operate through a G protein PLC pathway (Phaneuf *et al.*, 1993) promoted tyrosine phosphorylation. Pervanadate stimulated IP formation fourfold over control and this effect was significantly inhibited by phenylarsine oxide (PAO), a compound that inhibits tyrosine phosphorylation (Yanaga *et al.*, 1995) (Fig. 5). By contrast, the stimulatory effect of fluprostenol was not affected by PAO (Fig. 5). Furthermore, when cells were treated with PDBu, an activator of PKC, the PLC response to fluprostenol was almost abolished (Fig. 5), whereas the response to pervanadate was less affected. Activation of PKC has an inhibitory feedback effect predominantly on G protein-linked PLC- β activity (Biffen *et al.*, 1993; Carrasco *et al.*, 1996). Taken together, these results suggest that the FP receptor in granulosa cells signals through a PLC- β , rather than PLC- γ pathway.

Fig. 4. Detection of tyrosine-phosphorylated proteins in human granulosa cells. Cells were stimulated with fluprostenol (Flu; $1 \mu\text{mol l}^{-1}$), oxytocin (OT; $1 \mu\text{mol l}^{-1}$), or pervanadate (PV; a mixture of $400 \mu\text{mol}$ sodium orthovanadate l^{-1} and $2 \text{ mmol H}_2\text{O}_2 \text{ l}^{-1}$). Proteins were resolved by polyacrylamide gel electrophoresis and phosphotyrosine was revealed by western blotting and enhanced chemiluminescence (ECL) detection. The positions of the molecular mass standards (kDa) are indicated on the left. Results are from a representative experiment, and similar findings were obtained with cells from two other donors.

Fig. 5. Effects of (a) phenylarsine oxide (PAO) and (b) phorbol 12,13-dibutyrate (PDBu) on fluprostenol- and pervanadate-stimulated formation of [^3H]IPs in cultured human granulosa cells. Cells were labelled with [^3H]myo-inositol and were challenged with fluprostenol ($1\ \mu\text{mol l}^{-1}$) or pervanadate (a mixture of $400\ \mu\text{mol}$ sodium orthovanadate l^{-1} and $2\ \text{mmol H}_2\text{O}_2\ \text{l}^{-1}$) in the presence of LiCl. Cells were stimulated with agonists in the absence (control; □) or presence (■) of PAO ($1\ \mu\text{mol l}^{-1}$) or PDBu ($1\ \mu\text{mol l}^{-1}$), which were added 5 and 10 min before the agonists, respectively. Results are expressed as relative change and are the mean $\pm$ SD of quadruplicate determinations from single representative experiments. The findings were confirmed with cells from different donors. Statistical differences compared with cells without inhibitor: *, $P < 0.05$; **, $P < 0.01$.

Expression of G_{α_q} and $G_{\alpha_{11}}$ genes in human granulosa cells

For identifying the class of G protein involved in the mechanism of FP receptor activation, granulosa cells were pretreated with $0.5\ \mu\text{g}$ pertussis toxin ml^{-1} for 16 h before being challenged with fluprostenol. The effect of fluprostenol

Fig. 6. Effect of pertussis toxin on fluprostenol-stimulated PLC activation and changes in $[\text{Ca}^{2+}]_i$ in human granulosa cells. Cells were preincubated with (■) and without (□) $0.5\ \mu\text{g}$ pertussis toxin ml^{-1} for 16 h and were challenged with $0.1\ \mu\text{mol}$ fluprostenol l^{-1} . All other conditions were as in Fig. 2. Results are expressed as relative change and are the mean $\pm$ SD of quadruplicate determinations from a single representative experiment. The findings were confirmed with cells from two different donors.

was resistant to pertussis toxin (Fig. 6), indicating that it is mediated by a protein of the G_q family. Human granulosa cells have been shown to express G proteins of the G_q family as demonstrated by immunoblotting, although the antibody used could not distinguish between G_{α_q} and $G_{\alpha_{11}}$ (López Bernal *et al.*, 1995). Here, we have demonstrated the presence of mRNA for both G_{α_q} and $G_{\alpha_{11}}$ in granulosa cells. RT-PCR analysis using G_{α_q} - and $G_{\alpha_{11}}$ -specific primers that were designed to produce a 508 bp and a 529 bp fragment, respectively, resulted in the amplification of single bands (Fig. 7). The identity of the PCR products was confirmed by sequencing which showed 100% homology with the published sequences (Jiang *et al.*, 1991; Chen *et al.*, 1996).

Discussion

The data reported here show that activation of FP receptors in human granulosa cells with the selective agonist fluprostenol results in a dose-dependent stimulation of PLC and an increase in $[\text{Ca}^{2+}]_i$ concentrations and confirm that $\text{PGF}_{2\alpha}$ may be an important physiological modulator of ovarian activity (McNatty *et al.*, 1975; Thomas *et al.*, 1978; Hamberger *et al.*, 1979; Pate and Condon, 1984; Michael and Webley 1993) through a pathway that involves activation of the inositol phospholipid pathway. $\text{PGF}_{2\alpha}$ operates through the FP receptor which has recently been cloned from human uterine tissues. This receptor displays the characteristic seven-transmembrane domains of G protein-coupled receptors (Abramovitz *et al.*, 1994). Binding studies with radiolabelled $\text{PGF}_{2\alpha}$ indicated the presence of FP receptors in human luteal cells (Powell *et al.*, 1974; Auletta *et al.*, 1990; Olofsson and Leung, 1994), and recently, Ristimäki *et al.* (1997) have cloned a partial cDNA sequence of the FP receptor from human granulosa cells. We have demonstrated the presence of FP receptor mRNA in

Fig. 7. (a) Expression of $G\alpha_q$ gene in human granulosa cells. RT-PCR analysis of $G\alpha_q$ produced the expected 508 bp band (lanes 1, 2 and 4; three different donors). A PCR sample without RNA transcribed product was loaded in lane 3 (blank). Lane 5 contained the DNA size markers (bp). (b) Expression of $G\alpha_{11}$ gene in human granulosa cells. RT-PCR analysis of $G\alpha_{11}$ produced the expected 529 bp band (lane 2). A PCR sample without RNA transcribed product was loaded in lane 3 (blank). Lanes 1 and 4 contain DNA size markers. A sample of human myometrial cells was applied in lane 5 for comparison. The identity of the PCR products was confirmed by sequencing.

granulosa cells by RT-PCR using a different set of primers from those used by Ristimäki *et al.* (1997). Moreover, Pierce *et al.* (1997) have cloned two isoforms of the FP receptor from an ovine large luteal cell library, with minor differences in the carboxy terminus.

Furthermore, in the present study we have shown that human granulosa cells express three subtypes of PLC- β ($\beta 1$, $\beta 2$ and $\beta 3$) and two subtypes of PLC- γ ($\gamma 1$ and $\gamma 2$) raising the possibility that FP receptors operate through tyrosine kinase as well as G protein-coupled pathways (Watanabe *et al.*, 1994). In addition, we have demonstrated by RT-PCR that mRNAs for two pertussis toxin-resistant G proteins, $G\alpha_q$ and $G\alpha_{11}$, are present in abundance in cultured granulosa cells. Since G proteins of the G_q family activate PLC- β rather than PLC- γ isoforms (Rhee and Choi, 1992), it is suggested that in granulosa cells the FP receptor operates by increasing phosphoinositide formation and $[Ca^{2+}]_i$ through a G_q /PLC- β pathway.

This is supported by the fact that despite the high expression of PLC- γ in cultured human granulosa cells, there was not stimulation of tyrosine phosphorylation in cells treated with the G protein receptor agonists fluprostenol or oxytocin. By contrast, pervanadate produced a marked increase in protein phosphorylation. Moreover, in the PLC activation assays the pattern of response to fluprostenol was very different from the response to pervanadate. Stimulation of PKC by phorbol ester inhibited the response to fluprostenol much more than the response to pervanadate. PLC- β isoenzymes are very sensitive to PKC whereas the response to PLC- γ enzymes is not affected (Biffen *et al.*, 1993). In addition, PAO, which blocks tyrosine phosphorylation of several proteins (García-Morales *et al.*, 1990; Anel and Kleinfeld, 1993; Yanaga *et al.*, 1995), inhibited the response to pervanadate without having any effect on the response to fluprostenol. These results clearly suggest that fluprostenol operates through a PKC-sensitive pathway such as PLC- β that is not affected by blocking tyrosine phosphorylation and does not involve PLC- γ (Carrasco *et al.*, 1996). This contention is in agreement with the data published by Abayasekara *et al.* (1993) and Michael *et al.* (1993) who reported that the actions of PGF_{2u} on human and marmoset luteal cells involve a PKC-dependent mechanism.

FP receptors are hormonally regulated. In primates, the activation of PLC by PGF_{2u} is enhanced as the luteal phase progresses (Houmard *et al.*, 1992); however, by day 14 after ovulation the response to PGF_{2u} or its analogue cloprostenol is lost, suggesting that in the primate corpus luteum recent exposure to LH or hCG is necessary to stimulate PLC via FP receptors (Michael and Webley, 1993). In sheep, FP receptor transcripts are high during the mid-luteal phase but decrease during luteolysis (Graves *et al.*, 1995). A similar pattern has been described for bovine FP receptors which are maximally induced 48 h after the ovulatory LH surge (Sakamoto *et al.*, 1995; Tsai *et al.*, 1996). Interestingly, high levels of FP receptor transcripts are found in bovine corpus luteum during early pregnancy (Sakamoto *et al.*, 1995). Recent experiments in mice, suggest a role for FP receptors in luteal cell apoptosis (Hasumoto *et al.*, 1997). Moreover, Ristimäki *et al.* (1997) have shown that in human cultured granulosa cells FP receptor mRNA expression is stimulated by hCG through a cyclic AMP-dependent pathway and the effect of hCG was abolished with phorbol ester. Thus, activation of PKC not only inhibits the response of PLC- β to the receptor, but also leads to receptor downregulation. Stimulation of FP receptor expression by LH/hCG and inhibition by PKC (which can be activated by PGF_{2u} itself) may provide a fine balance to regulate the activity and lifespan of the corpus luteum. In conclusion, human granulosa cells express an FP receptor which is functional and activates PLC- β probably via a pertussis toxin-resistant G_q protein. Our results apply to mature granulosa cells obtained from stimulated IVF cycles after exposure to hCG *in vivo* and may not apply to granulosa cells at earlier stages of development or to late luteal cells. Further research is necessary to establish the role of FP receptors in the process of luteinization and luteolysis and to define the PLC- β subtypes involved. An essential role for FP receptor-induced luteolysis in the initiation of parturition has recently been shown in mice (Sugimoto *et al.*, 1997).

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