Characterization of adenylyl cyclases in cultured human granulosa cells

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Granulosa cells play an essential role in follicular development and formation of corpora lutea. Many functions of granulosa–lutein cells are controlled by activation of G protein-coupled receptors and the formation of cyclic AMP (cAMP) by adenylyl cyclase. There are at least nine mammalian adenylyl cyclase isoenzymes, which show different sensitivities towards other signalling systems. The aim of this study was to identify the types of adenylyl cyclase present in human granulosa cells and to investigate its functional regulation by G proteins, calcium and the protein kinase C and A pathways. Granulosa cells were obtained from women undergoing IVF. The cells were maintained in primary culture and they consistently expressed mRNA coding for adenylyl cyclase I, III, VI, VII and IX. The signals for adenylyl cyclase V and VIII were more variable among patients and there was no signal for adenylyl cyclase II. The expression of multiple adenylyl cyclase proteins was confirmed by immunocytochemistry with subtype-specific antibodies. The formation of cAMP in cultured cells was stimulated many times by hCG (EC_{50} value 4.2 iu ml^{-1}) and by prostaglandin E_2 (PGE_2 ; $EC_{50} = 0.75 \text{ } \mu\text{mol l}^{-1}$) in a concentration-dependent manner, thus confirming the presence of receptors coupled positively to G_s . The diterpene forskolin, which stimulates all isoforms of adenylyl cyclase except for adenylyl cyclase IX, increased cAMP formation to higher levels than hCG or PGE_2 . The strong stimulation by forskolin indicates that adenylyl cyclase IX is unlikely to be the major source of

cyclase activity in these cells. Basal and forskolin- or PGE_2 -stimulated adenylyl cyclase activity was amplified 1.5–2.0 times by phorbol-12,13-dibutyrate, indicating that protein kinase C-sensitive enzymes (for example, adenylyl cyclase types IV, V, VI or VII) may be active in the cells. In contrast, hCG-stimulated activity was inhibited ($76 \pm 6\%$) by phorbol ester. Stimulation of G_i with the α -adrenoceptor agonist clonidine inhibited hCG-induced cyclase activity. This finding indicates that adenylyl cyclase II and IV subtypes, which are stimulated by $\beta\gamma$ subunits released from G_i , are not predominant. Increases in intracellular free calcium concentrations by the ionophore A23187, the calcium-ATPase inhibitor thapsigargin or by fluprostenol, a selective prostanoid FP receptor agonist, which is known to open calcium channels in granulosa cells, or removal of calcium by EGTA, had no significant effects on basal or forskolin-stimulated formation of cAMP. These results indicate that subtypes adenylyl cyclase I, III and VIII, which are activated by calcium, and adenylyl cyclase V and VI, which are inhibited by calcium, are not dominant isoforms in granulosa–lutein cells. The protein kinase A inhibitor H89 had no effects on formation of cAMP; this finding rules out the involvement of adenylyl cyclase V and VI subtypes, which are subjected to negative feedback by protein kinase A. These results indicate that adenylyl cyclase VII is the dominant functional isoenzyme in human granulosa–lutein cells.

Introduction

Granulosa cells play an essential role in follicular development, oocyte maturation and formation of corpora lutea. Many functions of granulosa–lutein cells are controlled by gonadotrophins, prostaglandins and other agonists through the second messenger cAMP. The intracellular concentra-

tion of cAMP is controlled primarily at the level of synthesis; cAMP is formed from ATP by agonist-stimulated adenylyl cyclase (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1.). Recent studies have identified nine isoforms of adenylyl cyclase, which are expressed unevenly in different tissues (Taussig and Gilman 1995; Sunahara *et al.*, 1996; Hurley, 1999). These isoenzymes, designated adenylyl cyclase I–IX, have similar structures, with two integral membrane domains each constituted by six putative transmembrane stretches (Krupinski *et al.*, 1989) and an active site formed by the interplay of two cytosolic

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domains (Yan *et al.*, 1996). The catalytic core of mammalian adenylyl cyclases consists of a pseudosymmetric heterodimer composed of highly conserved portions of the cytosolic domains, namely regions C1a and C2a (Hanoune *et al.*, 1997; Yan *et al.*, 1997), which are able to bind one molecule of $G\alpha_s$, one molecule of forskolin and one molecule of ATP (Tesmer and Sprang, 1998). A soluble form of adenylyl cyclase has been constructed by linkage of C1a and C2a regions and this has helped in studies of the regulation of the enzyme (Taussig and Gilman, 1995; Dessauer and Gilman, 1996).

Adenylyl cyclase is stimulated by receptors that promote the dissociation of heterotrimeric G_s proteins. The released $G\alpha_s$ -GTP unit binds and activates the cytosolic catalytic domains of the enzyme (Hurley, 1999). Although every known adenylyl cyclase isoform is stimulated by $G\alpha_s$ -GTP (Taussig and Gilman, 1995; Sunahara *et al.*, 1996), the effects of other signalling factors are less uniform due to heterogeneous expression of cytosolic regulatory domains on the enzyme. Adenylyl cyclase isoforms have been classified into four groups according to their mode of regulation (Hanoune *et al.*, 1997). For example, group 1 isoforms (adenylyl cyclase subtypes I, III and VIII) are stimulated by calcium and calmodulin (Krupinski *et al.*, 1989; Choi *et al.*, 1992; Cali *et al.*, 1994), whereas group 3 isoforms (types V and VI) are inhibited by increases in intracellular calcium concentrations (Fagan *et al.*, 1998). Protein kinase C exerts a stimulatory effect on group 2 (adenylyl cyclase II, IV and VII) (Taussig and Gilman, 1995; Sunahara *et al.*, 1996) and group 3 (adenylyl cyclase V and VI) (Sunahara *et al.*, 1996) subtypes. In contrast, cAMP-dependent protein kinase A has a negative feedback effect on adenylyl cyclase V and VI (group 3) (Iwami *et al.*, 1995). The heterotrimeric protein G_i has dual effects: the $G\alpha_i$ -GTP complex inhibits adenylyl cyclase V and VI, whereas the $\beta\gamma$ subunits released from G_i inhibit adenylyl cyclase I. Moreover, adenylyl cyclase II, adenylyl cyclase IV and probably adenylyl cyclase VII are stimulated by $G\beta\gamma$ subunits (Hurley, 1999). Group 4 isoforms (at present only adenylyl cyclase IX has been characterized) are insensitive to both calcium and $G\beta\gamma$ subunits (Taussig and Gilman, 1995; Hanoune *et al.*, 1997) but are inhibited by the phosphatase calcineurin (Antoni *et al.*, 1998).

The subtype selective sensitivity towards other second messenger systems facilitates the 'fine tuning' of cAMP concentrations within cells expressing different isoforms of adenylyl cyclases. The 'cross talk' between separate regulatory systems is especially important in granulosa cells, in which functions controlled by the activation of the LH-hCG receptor, that operate through G_s and stimulate cAMP formation, may be modulated by paracrine factors produced locally (for example, prostaglandins acting through different second messenger systems).

The aim of the present study was to characterize adenylyl cyclase activity in human granulosa cells and to identify functional isoforms of adenylyl cyclase. An *in vitro* system

was used to culture human granulosa cells obtained from stimulated IVF cycles (López Bernal *et al.*, 1995). RT-PCR was used to determine the expression of mRNAs coding for adenylyl cyclase isoforms and the expression of adenylyl cyclase protein was demonstrated by immunocytochemistry. Furthermore, cAMP accumulation in cells stimulated by hCG, prostaglandin E_2 and forskolin under different regulatory conditions was measured.

Materials and Methods

Materials

[2,8- 3H]-adenosine-3'5'-cyclic phosphate was obtained from Amersham International Plc (Little Chalfont). Percoll, antibiotic antimycotic solution (penicillin, streptomycin and amphotericin B), hyaluronidase type IV-S, Trypan blue, vitamin E, sodium selenite, adenosine-3'5'-cyclic phosphate, 1,1,2-trichlorotrifluoroethane, tri-*n*-octylamine, EDTA, theophylline, indomethacin, prostaglandin E_2 and nifedipine were from Sigma Chemical Co. Ltd (Poole). Forskolin, clonidine-HCl, phorbol-12,13-dibutyrate, EGTA, staurosporine, thapsigargin, H89 and A23187 were from Calbiochem-Novachem (Beeston). hCG was from Serono Labs UK Ltd (Hertfordshire). Fluprostenol was supplied by R. Coleman (formerly at Glaxo, Stevenage). Tissue culture reagents were from Life Sciences BRL (Inchinnan). All other reagents were commercial preparations of the highest available purity.

Custom oligonucleotide primers that were designed to recognize human adenylyl cyclase sequences recorded in the GenBank database were obtained from Life Technologies Ltd (Paisley). Adenylyl cyclase IX primers were developed by J. Patterson (Antoni *et al.*, 1995, 1998). Omniscript™ RT Kit and Taq PCR Core Kit were purchased from Qiagen (Crawley). DNA molecular weight markers, 10 × TBE, Oligo p(dT)₁₅ and RNase inhibitor were obtained from Roche Diagnostics Limited (Lewes).

Specific polyclonal antibodies for adenylyl cyclase isoforms I, II, III, IV, V, VII and VIII, and their corresponding cognate peptides, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-adenylyl cyclase IX antiserum and cognate peptide were provided by F. Antoni (Antoni *et al.*, 1998). DAKO biotin blocking system, biotinylated swine anti-goat/mouse/rabbit Ig, rabbit anti-mouse IgG and streptavidin AP were purchased from DAKO Ltd (Ely). Naphthol AS-MX phosphate free acid, *N*, *N*-dimethylformamide, levamisole, Phe-Gly-Gly and fast red TR salt were supplied by Sigma-Aldrich Co Ltd. Aquamount was supplied by BDH Laboratory Supplies (Poole).

Preparation and culture of human granulosa cells

Granulosa cells were collected with informed consent from women undergoing routine retrieval of oocytes for IVF. Granulosa cells from women with endometriosis or

polycystic ovaries were excluded. The main indications for IVF were unexplained infertility, tubal damage and male factor infertility. Follicular growth was stimulated by daily injection of gonadotrophins in patients previously desensitized with a long course of GnRH (Al Azemi *et al.*, 2000). Follicles were aspirated 35 h after administration of 10 000 iu hCG (Profasi; Serono labs UK Ltd, Hertfordshire). After removal of any oocytes, cells were collected from follicular fluid by centrifugation at 340 *g*, washed with Hank's balanced salt solution (HBSS) and centrifuged at 340 *g* at room temperature for 30 min on 40% (v/v) Percoll. Granulosa cell layers were subsequently collected from the interface, washed in HBSS and dissociated using 1 mg hyaluronidase ml⁻¹. Viability of cells after dispersion was 80% as detected by Trypan blue staining and the yield per patient was 0.4–1.5 × 10⁶ cells. Cells were seeded in 24 well plates at 4.0 × 10⁴ cells cm⁻² and cultured in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 mix with glutamax (1:1 by volume) medium containing 10% (v/v) fetal calf serum, 10% (v/v) horse serum, 1 µmol vitamin E l⁻¹, 20 nmol sodium selenite l⁻¹, 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹ and 0.25 µg amphotericin B ml⁻¹. The cultures were kept at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Culture medium was exchanged daily and the cells were used within the first week in culture. 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).

RNA isolation

Total cellular RNA was prepared using a modification of the guanidine–isothiocyanate method (Chirgwin *et al.*, 1979). In brief, cells (approximately 10⁶) were solubilized in 3 ml of 4 mol guanidium isothiocyanate l⁻¹ containing 5 g sodium *N*-lauryl sarcosine l⁻¹, 0.7% (v/v) mercaptoethanol and 25 mmol sodium citrate l⁻¹ (pH 7.0), and layered onto an 8.5 ml cushion of 5.7 mol caesium chloride l⁻¹ containing 10 mmol Na₂EDTA l⁻¹ and sedimented at 175 000 *g* for 20 h at 20°C in a Beckman SW 41Ti-rotor. RNA concentrations were determined by UV absorption at 260 nm. The integrity of the 28 and 18S ribosomal RNA was assessed by agarose gel electrophoresis.

RT-PCR amplification

First strand cDNA synthesis using Omniscript™ RT. Total RNAs from human cultured granulosa cells were used as templates for first strand cDNA synthesis using Oligo-p(dT)₁₅ primer and Omniscript™ reverse transcriptase supplied in Omniscript™ RT kit. First strand cDNA synthesis was carried out in a 40 µl reaction volume containing 1 µg total RNA, 10 µmol Oligo-p(dT)₁₅ l⁻¹, 0.5 mmol deoxynucleoside triphosphates l⁻¹, 10 U RNase inhibitor and 4 U Omniscript™ reverse transcriptase. Transcription was carried out at 37°C for 1 h before storage at -70°C. Human brain total RNA was also prepared

as described above and used as a control tissue in RT-PCR.

Amplification of first strand cDNAs by PCR. Messenger RNAs encoding adenylyl cyclase isoforms I, II, VII, VIII, IX and for Gα_s were amplified as described by Price *et al.* (2000) using oligodeoxynucleotide primers based on human sequences published in the GenBank database. A HotStart PCR protocol was used to amplify adenylyl cyclase III. Additional primers were designed to amplify adenylyl cyclases V and VI using a partial sequence for *Homo sapiens* cardiac adenylyl cyclase types V and VI (Raimundo *et al.*, 1999). For adenylyl cyclase V: forward primer of 20 bases representing nucleotides 41–60 and a reverse primer of 20 bases complementary to nucleotides 231–212. For adenylyl cyclase VI: forward primer of 18 bases representing nucleotides 7–24 and a reverse primer of 20 bases complementary to nucleotides 197–178. Forty cycles of PCR were required to amplify mRNA for adenylyl cyclases V and VI. None of the primers used amplified a product from genomic DNA (data not shown).

Electrophoresis of PCR products. The PCR products from the amplification reaction were resolved using a 2% (w/v) agarose gel and the DNA bands were visualized by ethidium bromide staining and UV illumination. All primer pairs produced a single PCR product band of the expected size; however, primers designed to amplify cDNA coding for Gα_s produced a double banded product corresponding to Gα_s large and Gα_s small.

Sequencing of PCR products. The identities of the PCR products obtained were confirmed by Dye Terminator Sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Oxford, Oxford) using the primers designed for each isoform of adenylyl cyclase.

Immunocytochemistry

Granulosa cells were cultured on glass coverslips for 48 h, fixed with acetone for 10 min, rinsed in distilled water and Tris-buffered saline (TBS) for 5 min, and then incubated in avidin solution followed by biotin solution for 10 min to block endogenous biotin and to inhibit non-specific background staining. The cells were incubated for 30 min with primary antibody diluted optimally to 2 µg ml⁻¹ in TBS, washed and incubated for a further 30 min with biotinylated secondary antibody diluted 1:100 in TBS. The cells were incubated for 30 min with streptavidin (1:200 in TBS) followed by 30 min incubation with Fast Red Substrate (1 mg Naphthol AS-MX phosphate free acid dissolved in 0.1 ml *N,N*-dimethylformamide and added to 4.9 ml of 0.1 mol Tris l⁻¹ (pH 8.2) containing 5 µl of 1 mol levamisole l⁻¹, 50 µl of 100 mmol Phe-Gly-Gly l⁻¹ and 5 mg Fast Red TR salt). Control reactions included IgG instead of the antibodies or antibodies preabsorbed with their cognate peptides. Coverslips were counterstained

with haematoxylin and mounted onto glass slides using Aquamount.

Measurement of intracellular cAMP

The cells were washed with HBSS for 10 min at 37°C and preincubated with the phosphodiesterase inhibitor theophylline (5 mmol l⁻¹) and the prostaglandin synthesis inhibitor indomethacin (10 µmol l⁻¹) in HBSS for 10 min. In several experiments the preincubation mixture also contained other specific enzyme inhibitors, as described in the results section. All compounds were dissolved initially in ethanol or dimethylsulphoxide (DMSO), and solvent controls were included in the experiments. Agonists were added in 0.5 ml HBSS and the cells were incubated at 37°C for 10 min in the presence of theophylline and indomethacin. The reaction was stopped by replacing the incubation fluid with 0.5 ml ice-cold 5% (v/v) perchloric acid. The cells were scraped, centrifuged for 10 min at 1000 g and the acid soluble fraction was neutralized by the addition of 1.3 volumes of 1,1,2-trichlorotrifluoroethane: tri-*n*-octylamine (1:1, v/v). The mixture was shaken intensively for 2 min, centrifuged at 1000 g for 10 min and the upper phase was stored at -20°C until used for cAMP estimations. cAMP was measured by the competitive protein-binding assay of Brown *et al.* (1971) as described by López Bernal *et al.* (1991) using [³H]cAMP as tracer. Radioactivity was measured in 5 ml Wallac OptiPhase 'HiSafe' scintillation cocktail (Fisons, Loughborough) using a Beckman LS 5000 liquid scintillation counter. Values were expressed in d.p.m., taking into account counting efficiency (typically 40–51% for ³H). All cAMP estimations were performed in quadruplicate. Data were plotted and analysed using Prism 2.01 (Graphpad Software Inc, San Diego, CA).

Protein measurement

Protein was determined by the bicinchoninic acid method using a commercially available kit (Pierce, Rockford, IL).

Results

Expression of adenylyl cyclase isoforms in granulosa cells

The results of the mRNA analysis are shown (Fig. 1). Cells from four different donors were studied and there was consistent expression of mRNA coding for adenylyl cyclase I, III, VI, VII and IX. The signals for adenylyl cyclase V and VIII were more variable among patients and there was no apparent signal for adenylyl cyclase II, although the message for this isoform was detected clearly in brain tissue. The quality of the PCR technique was confirmed by the detection of message for Gα_s large and Gα_s small as expected (Europe-Finner *et al.*, 1997; Price *et al.*, 2000).

Staining of granulosa cells with specific adenylyl cyclase antibodies confirmed the mRNA data and revealed the presence of several adenylyl cyclase proteins in the cells, including group 1 (adenylyl cyclase I, III and VIII), group 2 (adenylyl cyclase IV and VII), group 3 (adenylyl cyclase V) and group 4 (adenylyl cyclase IX) isoforms (Fig. 2). There was no staining for adenylyl cyclase II, thereby confirming the PCR data. In all cases the staining was virtually abolished by pre-incubating the antibodies with their cognate peptide, confirming the specificity of the reaction.

Receptor-stimulated adenylyl cyclase activity

The accumulation of cAMP in cultured human granulosa cells was stimulated, in a concentration dependent manner, by hCG and PGE₂ up to 50 times compared with basal cAMP content (Fig. 3). The EC₅₀ values were 4.2 ± 0.1 iu hCG ml⁻¹ (*n* = 3) and 0.75 ± 0.18 µmol PGE₂ l⁻¹ (*n* = 7). The diterpene forskolin increased cAMP formation, but although this effect was concentration dependent, obvious signs of saturation were not observed at the highest concentration used (100 µmol l⁻¹). These results confirm that there is adenylyl cyclase activity in cultured granulosa cells and that it is functionally activated by two independent receptors coupled to Gα_s.

Effect of protein kinases

Basal, forskolin- and PGE₂-stimulated formation of cAMP was amplified by the protein kinase C activator phorbol-12,13-dibutyrate (Fig. 4). In contrast, hCG-stimulated formation of cAMP was inhibited strongly by phorbol-12,13-dibutyrate. This inhibition was reversed by the protein kinase C inhibitor staurosporine (1 µmol l⁻¹). The selective cAMP dependent protein kinase (protein kinase A) inhibitor H89 had no effect on forskolin-, hCG- or PGE₂-stimulated formation of cAMP (Fig. 5).

Effect of G_i activation

When granulosa cells were incubated with clonidine, which signals through α-adrenoceptors coupled to G_i (Stevens and Pyne, 1995; López Bernal *et al.*, 1995), hCG-stimulated cAMP formation was inhibited significantly (Fig. 6). The IC₅₀ value of clonidine was 1.9 ± 1.1 nmol l⁻¹ (*n* = 3).

Role of calcium

Increasing Ca²⁺ concentrations using the ionophore A23187 (10 µmol l⁻¹), the calcium-ATPase inhibitor thapsigargin (100 nmol l⁻¹) or the stable prostaglandin analogue fluprostenol (0.1–1.0 µmol l⁻¹), which is known to open calcium channels in granulosa cells (Carrasco *et al.*, 1997), had no significant effect on basal or forskolin-stimulated formation of cAMP. Chelating extracellular calcium with 3 mmol EGTA l⁻¹ also had no effect on basal or forskolin-stimulated formation of cAMP (Fig. 7 and data not shown).

Discussion

This is the first comprehensive molecular and functional characterization of adenylyl cyclase in human granulosa-lutein cells. The results of mRNA analysis and protein immunocytochemistry indicate that there are multiple isoforms of adenylyl cyclase in granulosa cells. Adenylyl cyclases are ubiquitous enzymes but they exhibit a degree of tissue and receptor selectivity that allows cell- and agonist-specific responses. Inclusion of the broad-spectrum phosphodiesterase inhibitor theophylline in the incubation system used in the present study ensured that any potential effects on the catabolism of cAMP were neutralized. Therefore, the observed changes in cAMP accumulation represented changes in adenylyl cyclase activity. The formation of cAMP in granulosa cells was clearly stimulated by hCG and PGE₂, which act through G protein-coupled receptors. Receptor activation promotes the dissociation of GDP from G α_s subunits and its replacement by the more abundant GTP, followed by the release of G α_s -GTP from the G $\beta\gamma$ subunit dimer. Active (GTP bound) G α_s binds to adenylyl cyclase, thus promoting the association of C1a and C2a and the formation of the active centre on adjoining faces of these cytosolic domains (Yan *et al.*, 1997).

Forskolin also promotes the formation of the catalytic core of most mammalian adenylyl cyclases by binding to C1a and C2a at sites different from those involving G α_s (Yan *et al.*, 1998). However, forskolin did not appear to stimulate mouse adenylyl cyclase IX, when it was expressed heterologously in Sf9 cells (Premont *et al.*, 1996) or when the cytosolic domains of adenylyl cyclase IX were expressed in *Escherichia coli* (Yan *et al.*, 1997). The soluble form of adenylyl cyclase IX is functional, can be stimulated by G α_s and was rendered forskolin-sensitive by mutating Tyr1082 to Leu (Yan *et al.*, 1998). Very moderate stimulation by forskolin was observed when adenylyl cyclase IX was expressed in HEK293 cells (Premont *et al.*, 1996) or studied in the AtT20 tumour cell line (Antoni *et al.*, 1995). In the present study, forskolin proved to be a very effective stimulator of adenylyl cyclase activity compared with hCG and PGE₂, indicating that adenylyl cyclase IX is not the single most active isoform in human granulosa cells.

Protein kinase C has a complex role in the regulation of granulosa-lutein cells, which may be mediated, in part, by effects on cAMP production. Protein kinase C activation stimulates progesterone production in bovine (Brunswick *et al.*, 1986) and human granulosa-lutein cells (Jalkanen *et al.*, 1987). In contrast, protein kinase C inhibits acute (Abayasekara *et al.*, 1993) and long-term (Ristimäki *et al.*, 1997) effects of hCG in cultured human granulosa-lutein cells. In the present study, activation of protein kinase C using phorbol dibutyrate enhanced basal, forskolin- and PGE₂-stimulated accumulation of cAMP. As forskolin activates adenylyl cyclase without the intervention of G proteins, these results indicate that protein kinase C has a direct effect on enzyme activity. These results support

Fig. 1. Identification of human granulosa adenylyl cyclase (AC) and G α_s cDNAs. RT-PCR was performed and the cDNAs were resolved on a 2% (w/v) agarose gel and visualized by ethidium bromide staining and subsequent UV illumination. Lane 1: negative control, reverse transcription performed using water rather than RNA, with subsequent PCR. Lanes 2, 3, 4 and 5: granulosa cell cDNA from four different donors. Lane 6: human brain cDNA as positive control.

those of Jalkanen *et al.* (1987) and Wheeler and Veldhuis (1989), who found that phorbol esters facilitated forskolin-stimulated generation of cAMP in freshly isolated human and pig luteal cells, respectively. In contrast, other groups have found that phorbol ester treatment has no effect on accumulation of cAMP in primary cultures of rat (Shinohara *et al.*, 1985; Davis *et al.*, 1989a) or bovine luteal cells (Davis 1992) stimulated with cholera toxin or

Fig. 2. For legend see facing page.

Fig. 2. Immunocytochemical demonstration of adenylyl cyclases in cultured human granulosa cells using isotype-specific antibodies (left-hand column). Cell staining was performed by the avidin–biotin method using Fast Red substrate. Corresponding control reactions using antibodies preabsorbed with their cognate peptides are shown in the right-hand column. Scale bars represent 50 μ m.

Fig. 3. Effects of hCG (■), PGE₂ (●) and forskolin (□) on formation of cAMP in cultured human granulosa cells. Cells were incubated in medium containing 5 mmol theophylline l⁻¹ and 10 μmol indomethacin l⁻¹ and were stimulated with hCG, PGE₂ or forskolin for 10 min at 37°C. The reaction was stopped by adding cold perchloric acid and cAMP was measured from the acid soluble supernatant. Each data point represent the mean ± SEM of four parallel incubations, using cells pooled from three patients. Similar results were found in another experiment, using cells pooled from four patients (data not shown).

forskolin. Protein kinase C exerts isotype-specific effects on isoforms of adenylyl cyclase. Phorbol ester treatment increased basal and G α_s -stimulated activity of the heterologously expressed adenylyl cyclase II, with a parallel increase in phosphorylation of the enzyme protein (Jacobowitz and Iyengar 1994). Similarly, basal and forskolin-stimulated formation of cAMP was enhanced with adenylyl cyclase II, VII and V (Jacobowitz *et al.*, 1993; Watson *et al.*, 1994; Kawabe *et al.*, 1994). Receptor and G α_s -stimulated, but not basal or forskolin-stimulated, formation of cAMP was increased by phorbol ester treatment in HT4 cells expressing adenylyl cyclase I and VI (Morimoto and Koshland, Jr, 1994). The results of the present study, which indicate that protein kinase C activation has a stimulatory effect, also indicate that isoforms of adenylyl cyclase, such as adenylyl cyclase II, IV, V, VI or VII, are probably present in granulosa cells. However, another layer of complexity may arise from the fact that cells express different isoforms of protein kinase C, which may have different effects on the activation of adenylyl cyclase (Kawabe *et al.*, 1994). This fact may explain the apparently contradictory results. For example, adenylyl cyclase V and VI, expressed in HEK 293 cells, remained unaffected by

Fig. 4. Effects of protein kinase C activation on formation of cAMP in cultured human granulosa cells. Cells were incubated in buffer containing 5 mmol theophylline l⁻¹ and 10 μmol indomethacin l⁻¹, and were stimulated with 1 μmol forskolin l⁻¹, 10 iu hCG ml⁻¹ or 1 μmol PGE₂ l⁻¹ for 10 min at 37°C with and without 1 μmol phorbol-12,13-dibutyrate l⁻¹ or phorbol-12,13-dibutyrate plus 1 μmol staurosporin l⁻¹. The reaction was stopped by the addition of cold perchloric acid and cAMP was measured from the acid soluble supernatant. Data are shown as relative stimulation by phorbol-12,13-dibutyrate versus untreated cells. Values are mean ± SEM of three experiments, using cells obtained from different patients. Note that phorbol-12,13-dibutyrate enhanced basal, forskolin- and PGE₂-activated adenylyl cyclase activity, but depressed hCG-stimulated activity very significantly (**P* < 0.01). The effect of phorbol-12,13-dibutyrate was blocked completely by staurosporin.

phorbol ester treatment (Jacobowitz *et al.*, 1993) but were highly stimulated when treated with protein kinase C in a purified form (Kawabe *et al.*, 1994). Nevertheless, the moderate activation observed in the present study does not support the involvement of adenylyl cyclase V in human granulosa cells, as adenylyl cyclase V is activated directly five to 20 times by at least two isoforms of protein kinase C (Kawabe *et al.*, 1994).

Human granulosa cells express functional PGF_{2α} receptors positively coupled to phospholipase C (Carrasco *et al.*, 1997). However, in our experiments, fluprostenol had no effect on accumulation of cAMP, although stimulation of protein kinase C by diacylglycerol liberated as a consequence of activation of phospholipase C would be expected. Perhaps the level of protein kinase C stimulation achieved with phorbol esters exceeds the stimulation from transient increases in diacylglycerol induced by fluprostenol. Similarly, in bovine luteal cells, phorbol ester treatment inhibited LH-induced activation of phospholipase C, but PGF_{2α} had no effect (Davis, 1992). PGF_{2α} and cloprostenol inhibited hCG-stimulated accumulation of cAMP in cultured human granulosa-lutein cells (Davis *et al.*, 1989b; Michael and Webley, 1991; Abayasekara *et*

Fig. 5. Effects of the protein kinase A inhibitor H89 on formation of cAMP in granulosa cells. Cells were incubated in buffer containing 5 mmol theophylline l⁻¹ and 10 µmol indomethacin l⁻¹ with (■) and without (□) 10 µmol H89 l⁻¹. Cells were stimulated with 1 µmol forskolin l⁻¹, 10 iu hCG ml⁻¹ or 1 µmol PGE₂ l⁻¹ for 10 min at 37°C. The reaction was stopped by the addition of 500 µl ice cold perchloric acid and cAMP was measured from the acid soluble supernatant. Values are mean ± SEM of four estimations in a representative experiment. Similar results were obtained in two other experiments using cells from different patients (data not shown).

Fig. 6. Effects of clonidine on hCG-stimulated formation of cAMP in cultured human granulosa cells. Cells were incubated in buffer containing 5 mmol theophylline l⁻¹, 10 µmol indomethacin l⁻¹ and 10 iu hCG ml⁻¹ for 10 min at 37°C with different concentrations of clonidine. Control incubations contained no clonidine. The reaction was stopped with perchloric acid, and cAMP was measured in the acid soluble supernatant. Data are shown as percentages of maximal stimulation. Values are mean ± SEM of three different experiments. *Value is significantly different from control ($P < 0.05$).

Fig. 7. Effects of changes in calcium concentration on formation of cAMP in granulosa cells. Cells were incubated in buffer containing 5 mmol theophylline l⁻¹ and 10 µmol indomethacin l⁻¹ and were stimulated with 1 µmol forskolin l⁻¹ for 10 min at 37°C. Calcium concentration was influenced by the addition of 5 µmol A23187 l⁻¹ or 0.1 µmol fluprostenol l⁻¹. The reaction was stopped by the addition of cold perchloric acid and cAMP was measured in the acid soluble supernatant. Values are mean ± SEM of four incubations in a representative experiment. Similar results were obtained in two other experiments using cells from different patients (data not shown).

al., 1993) and in marmoset monkey luteal tissue (Michael and Webley, 1993), but remained ineffective in cells preincubated overnight with hCG or other agents that increase cAMP content (Michael and Webley, 1991).

Adenylyl cyclase activity can also be subjected to negative feedback by cAMP-sensitive protein kinase (protein kinase A). Both type V and VI sequences have a conserved putative protein kinase A phosphorylation site at the C1a domain near the transmembrane span 7. This site is not expressed in other adenylyl cyclases (Premont *et al.*, 1992). Protein kinase A phosphorylates and inactivates adenylyl cyclase types V and VI directly (Iwami *et al.*, 1995). In the present study, the selective protein kinase A inhibitor H89 (Chijiwa *et al.*, 1990) had no effect on basal or hCG- and forskolin-stimulated adenylyl cyclase activity. This observation is in accordance with a low functional activity of adenylyl cyclase V and VI in human granulosa cells.

Alpha-adrenoceptors coupled to G_i potentially have several effects on adenylyl cyclase activity. Receptor activation leads to the release of Gα_i and Gβγ subunits. Gα_i has inhibitory effects on adenylyl cyclase I, V and VI, whereas Gβγ subunits inhibit adenylyl cyclase I but stimulate adenylyl cyclase II and IV. Under the conditions used in the pre-

sent study, activation of α -adrenoceptors by clonidine had a negative effect on hCG-stimulated accumulation of cAMP. The lack of any apparent stimulatory or additive effects of clonidine is indicative of a low activity of G $\beta\gamma$ -responsive adenylyl cyclase II and IV in granulosa cells.

Increasing calcium concentrations using the ionophore A23187, the calcium-ATP-ase inhibitor thapsigargin and fluprostenol, which open calcium channels in granulosa cells (Carrasco *et al.*, 1997), or chelation of calcium by EGTA, had no significant effects on basal and forskolin-stimulated formation of cAMP. These results indicate that subtypes adenylyl cyclase I and VIII, which are activated (Krupinski *et al.*, 1989; Choi *et al.*, 1992; Cali *et al.*, 1994), or adenylyl cyclase V and VI, which are inhibited directly by increases in calcium concentration (Fagan *et al.*, 1998), are not dominant in human granulosa cells. Calcium also exerts indirect inhibitory effects on adenylyl cyclase III via calcium-calmodulin dependent protein kinase II (Wei *et al.*, 1996), whereas adenylyl cyclase IX is inhibited by calcineurin (a calcium-sensitive protein phosphatase 2B) (Antoni *et al.*, 1995). Ford *et al.* (1996) demonstrated that calcineurin is present in rat luteal cells. The lack of effect of calcium manipulation in the present study is evidence against a functional involvement of adenylyl cyclase III and IX in human granulosa-lutein cells. This contention is in contrast to the bovine corpora lutea, in which calcium has a clear enhancing effect on forskolin-stimulated production of cAMP, implicating that adenylyl cyclase III is involved (Mamluk *et al.*, 1999).

Under the experimental conditions used in the present study, hCG-stimulated adenylyl cyclase activity was attenuated strongly by phorbol ester treatment, confirming previous observations by Davis *et al.* (1989b) and Abayasekara *et al.* (1993). Moreover, phorbol ester treatment suppressed FSH-induced adenylyl cyclase activity in rat granulosa cells (Shinohara *et al.*, 1985). In contrast, PGE₂-stimulated adenylyl cyclase activity was enhanced by phorbol esters in the present study. This observation highlights possible differences in the coupling of gonadotrophin and prostaglandin receptors to distinct G_s-adenylyl cyclase combinations. There are several G α_s variants in granulosa cells with potential phosphorylation sites (Europe-Finner *et al.*, 1997), which may be targets for regulation by protein kinase C. Moreover, the receptors themselves may be targets for phosphorylation. Further research should be aimed at identifying receptor-specific combinations of G protein and isoforms of adenylyl cyclase. Such studies will increase our understanding of the responses of ovarian cells to gonadotrophins, prostaglandins and other regulatory factors that operate through the cAMP pathway, and the cross-talk involvement of other signalling pathways.

The results of the present study demonstrate that there is adenylyl cyclase activity in cultured human granulosa cells that is stimulated strongly by physiological agonists, such as hCG and PGE₂, and by forskolin. Adenylyl cyclase activity is insensitive to changes in calcium concentra-

tions, G $\beta\gamma$ subunits or protein kinase A inhibitors, but is enhanced by co-activation of protein kinase C. Collectively, these data indicate that the main source of adenylyl cyclase activity in human granulosa-lutein cells is probably adenylyl cyclase VII, an adenylyl cyclase subtype that is expressed widely in mammalian tissues (Watson *et al.*, 1994). Moreover, the presence of mRNA and protein for several isoforms of adenylyl cyclase in human granulosa cells confirms findings in other human and rat tissues (Mhaouty-Kodja *et al.*, 1997; Emala *et al.*, 1998; Leech *et al.*, 1999; Price *et al.*, 2000). This apparent molecular redundancy provides detailed regulatory options and is a mechanism for integrating the responses to various endocrine and paracrine inputs when the functional expression of gonadotrophin, prostanoid and other receptors changes in ovarian cells. These studies should be extended to granulosa and theca cells at different stages of follicular development, and to late luteal cells, to further our understanding of the essential role of the cAMP signalling pathway in ovarian function.

This work was supported by Wellbeing (grant reference L1/96). The authors are grateful to the staff at the Oxford Fertility Unit for their help in the collection of granulosa cells. The authors would like to thank F. Antoni for providing anti-adenylyl cyclase IX antibody and J. M. Paterson for the adenylyl cyclase IX PCR primers.

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Received 10 April 2000.

Accepted 7 September 2000.