Changes in cAMP-dependent protein kinase (PKA) and progesterone secretion in luteinizing human granulosa cells

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Abstract

Luteinization of follicular granulosa cells leads to an increase in progesterone secretion that is regulated by luteinizing hormone (LH). LH acts mainly by elevating intracellular cyclic 3',5'-adenosine monophosphate (cAMP) and activating cAMP-dependent protein kinase (PKA). In this study, we have examined the role of PKA in relation to progesterone output by luteinizing human granulosa cells. Human granulosa cells were obtained by percoll gradient centrifugation of follicular aspirates of patients undergoing oocyte retrieval for assisted conception. Cells were cultured in serum-supplemented medium for up to 3 days in the presence and/or absence of human (h)LH and other cAMP-elevating agents. Spent medium was assayed for cAMP and progesterone content by specific RIA. Cell lysates were collected and assessed for PKA regulatory (R)II α /catalytic (C) α expression by Western blotting. Although basal progesterone secretion

increased progressively throughout culture, cAMP levels remained unchanged. Under basal conditions, PKA RII α /C α expression appeared to increase throughout the 3-day culture period. In the presence of hLH and other cAMP-elevating agents, progesterone secretion increased in a dose-dependent manner coincident with an increase in cAMP. However, despite the increase in both progesterone secretion and cAMP accumulation, there was a dose-dependent decrease in both PKA RII α and C α expression. Thus, data presented in this study show that increases in progesterone secretion in luteinizing human granulosa cells can be dissociated from increases in PKA expression. This notion implies that progesterone secretion may be regulated by PKA-dependent as well as PKA-independent mechanisms.

Journal of Endocrinology (2004) **183**, 39–50

Introduction

Following the surge in luteinizing hormone (LH) and ovulation, follicular remnants are transformed into the corpus luteum (CL) through the luteinization of granulosa and theca cells. This is an event essential to the establishment and maintenance of pregnancy (Spencer & Bazer 2002). It is generally accepted that binding of LH to its G-protein-coupled receptor leads to the activation of the adenylate cyclase/cyclic-AMP (cAMP)/protein kinase A (PKA) signalling pathway (Marsh & Savard 1966, Marsh 1970, Leung & Steele 1992, Davis 1994). Thus, LH exerts its actions on progesterone synthesis primarily by elevating intracellular cAMP levels, leading to the activation of PKA, the activity of which has been shown to influence the expression (Strauss *et al.* 1999, Clark *et al.* 2001) or activity (Sugawara *et al.* 1995) of components of the steroidogenic pathway. Moreover, it has been shown that LH-induced gonadal steroidogenesis can also be regulated through cAMP/PKA-independent mechanisms (Cooke 1999, Wood & Strauss 2002).

The diversity of PKA and its subunits has been extensively reviewed (Dosekand *et al.* 1993, Skallhegg & Tasken 2000). In the absence of cAMP, the PKA holoenzyme exists as a tetramer composed of two regulatory (R) and two catalytic (C) subunits. When both binding sites on the R subunits are occupied by cAMP, the R subunits undergo a conformational change, which lowers their affinity for the C subunits. This results in the dissociation of the holoenzyme complex and renders the enzyme active (Skallhegg & Tasken 2000). The catalytic subunits are then free to phosphorylate specific target proteins.

To date, four regulatory subunits have been identified (RI α , RI β , RII α and RII β), which are differentially distributed in mammalian tissues (Skallhegg & Tasken 2000). RI α and RII α are expressed ubiquitously, RI β is expressed predominantly in the brain and RII β is expressed primarily in brain, adrenal and adipose tissues (Edelman *et al.* 1987, Taylor *et al.* 1990). These R subunits define types I and II PKA, with both types of holoenzyme having three potential C subunits (α , β and γ). The C α and β subunits share 93% homology and have broad tissue

specificity, with C α being the predominant species. The C γ subunit, however, has been readily identified only in the primate testis (Beebe *et al.* 1990).

Previous studies in the rat ovary have shown that RII is a major cytosolic phosphoprotein whose content and phosphorylation state are regulated by cAMP (Ratoosh & Richards 1985). It has been shown in the porcine ovary that type I PKA is present in equivalent amounts in small follicles, large preovulatory follicles and CL. However, type II PKA is present in much greater amounts, with a moderate increase in RII expressed in preovulatory follicles relative to small follicles and CL (DeManno & Hunzicker-Dunn 1991). Such reports suggest that type II, but not type I PKA, is hormonally regulated as a function of follicular differentiation in developing follicles. However, it has been reported that both types I and II PKA play a role in the regulation of steroidogenesis in other tissues (Whitehouse & Abayasekara 1994).

There is further evidence to suggest that PKA may play a role in the process of luteinization. For example, in the primate ovary, luteinization is accompanied by dramatic changes in the responsiveness of luteal cells to LH and cAMP (Zelevnik 1998, Zelevnik & Somers 1999). Moreover, in the rat ovary, it has been shown that inhibition of PKA inhibits morphological and physiological changes associated with luteinization (Morris & Richards 1993, 1995). Therefore, in this study, we have investigated the changes in the cAMP/PKA system as human granulosa cells undergo luteinization and acquire the capacity to secrete large quantities of progesterone.

Materials and Methods

Materials

Human luteinizing hormone (hLH; NIDDK-hLH-B-SIAFP-2 (6100 IU/mg)) was kindly donated by AF Parlow of the National Institute of Diabetes, Digestive and Kidney Diseases, National Hormone and Pituitary Program (Bethesda, MD, USA). Progesterone antibody was purchased from the Central Veterinary Laboratory (Reading, UK). [3 H]Progesterone, [125 I]cAMP-TME and Hyperfilm ECL were purchased from Amersham. cAMP standard and antibody were purchased from Calbiochem (Nottingham, UK). Protogel and SDS tank buffer were purchased from National Diagnostics (Hull, UK). Rabbit polyclonal anti-PKA C α and rabbit polyclonal anti-PKA RII α were purchased from Santa Cruz (Calne, UK). Monoclonal mouse antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Biogenesis Ltd (Poole, UK). Goat antirabbit IgG and bichinchoninic acid (BCA) reagent were purchased from Pierce (Chester, UK). Goat antimouse IgG was purchased from BD Transduction Laboratories (Cowley, UK). All other materials were purchased from Sigma (Poole, UK) or BDH (Poole, UK) at the equivalent of Analar grade.

Patient samples

Human granulosa cells were obtained from follicular aspirates of patients undergoing oocyte retrieval for assisted conception at Lister Hospital, London, UK, with informed patient consent (in accordance with the Declaration of Helsinki) and with approval of the local ethics committee. Pituitary downregulation was achieved by subcutaneous administration of a GnRH analogue (Suprecur; Shire Pharmaceuticals, Andover, UK; 500 μ g/day from day 2 of the cycle for 10–21 days). Administration of the GnRH analogue was then continued in conjunction with purified urinary human menopausal gonadotrophin (Menogon; Ferring Pharmaceuticals, Feltham, UK; 2–4 ampoules daily for 10–14 days) followed by a single injection of human chorionic gonadotrophin (Profasi; Serono, Welwyn Garden City, UK; 5000–10 000 IU) 36 h prior to oocyte collection in which follicles were aspirated under local anaesthesia via the transvaginal route.

Isolation of human granulosa cells

Human granulosa cells were isolated as previously described (Webley *et al.* 1988, Abayasekara *et al.* 1993a). Follicular aspirates from individual patients were centrifuged at 250 *g* for 10 min at 4 °C. The supernatant was then aspirated, and cell pellets from individual patients were pooled and resuspended in phosphate-buffered saline (PBS), overlaid onto a 60% (v/v) Percoll solution and centrifuged at 1000 *g* for 20 min at 4 °C. Granulosa cells precipitated at the Percoll–PBS interface were aspirated, resuspended in PBS and centrifuged at 250 *g* at 4 °C. This step was repeated three times. After the final wash, the supernatant was removed and the cell pellet was resuspended in PBS. Cell viability was determined by the trypan blue (0.2% v/v) dye exclusion method. The number of steroidogenic cells determined by Δ^5 - 3β -hydroxysteroid dehydrogenase: Δ^5 - 4 -isomerase (3β HSD) cytochemistry (Aldred & Cooke 1983) was routinely found to be in excess of 85%.

Culture of human granulosa cells

Depending on experimental procedure, cells were plated in either 96-well plates or 35 mm culture dishes. For the measurement of progesterone and cAMP, cells were plated at a density of 2×10^4 cells per well/200 μ l medium in 96-well plates. For immunological detection of PKA C α or PKA RII α , cells were cultured in either individual 35 mm dishes or 6-well plates at a density of 10^6 cells/3 ml medium. Granulosa cells were cultured for up to 3 days in Dulbecco's modified Eagle's medium nutrient mixture F12 (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine and 1 IU/ml penicillin-streptomycin, and incubated at 37 °C in a humidified atmosphere with 95% O $_2$ and 5% CO $_2$.

Progesterone RIA

Progesterone content in the incubation medium was determined by an adaptation of the method previously described (Pallikaros *et al.* 1995). Prior to assay, samples were thawed, and diluted in assay buffer (20 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 80 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 150 mM NaCl, 1% (w/v) NaN_3 , 0.1% (w/v) gelatine) such that they lay within the linear portion of the standard curve. The intra- and interassay coefficients of variation were 9.4% and 10.3% respectively.

cAMP RIA

Medium collected from cell cultures was acidified with 3 M perchloric acid and stored at -20°C to await analysis by RIA. Prior to assay, samples were thawed and neutralized by the addition of 2.16 M K_3PO_4 , to give a final concentration of 0.154 M. cAMP concentrations were determined by specific radioimmunoassay by the method of Steiner *et al.* (1972), modified by the acetylation procedure of Harper and Brooker (1975), and validated for human granulosa cell-conditioned media by Abayasekara *et al.* (1993b). The intra- and interassay coefficients of variation were 10.9% and 12.4% respectively.

Preparation of luteinizing human granulosa cell lysates

After treatment of granulosa cells, cell lysates were prepared as previously described by Dewi *et al.* (2002). Incubation medium was aspirated from the dish, and the cells were rinsed with ice-cold PBS (pH 7.5). The wells of the culture dish were aspirated to dryness, and then lysis buffer (63.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% SDS, 1 mM AEBSEF [4-(2-aminoethyl) benzenesulfonyl fluoride] and 50 µg/ml leupeptin) was added to the cells, which were then left on ice for 10 min. Next, the luteinizing granulosa cells were scraped from the culture dish into 1.5 ml microfuge tubes. Cell lysates were then boiled at 100°C for 5 min. Protein content was determined by the BCA method, according to the manufacturer's instructions, and bromophenol blue and β -mercaptoethanol were added to the samples to give final concentrations of 0.02% (w/v) and 5% (v/v) respectively. Samples were then stored at -20°C until required, when they were thawed, reboiled for 5 min at 100°C and centrifuged at 13 000 r.p.m. for 1 min before electrophoresis.

SDS-PAGE and Western immunoblotting

Whole granulosa-cell lysates proteins were separated by 10% SDS-PAGE (Wheeler-Jones *et al.* 1996). After electrophoresis, proteins were transferred to Immobilon-polyvinylidene difluoride (PVDF) membrane by the BioRad semidry blotting system. After transfer of proteins to

a PVDF membrane, non-specific sites were blocked by a solution of Tris-buffered saline with Tween (TBS-T: 50 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20, pH 7.4), containing 10% (w/v) BSA, for 2 h at room temperature with gentle agitation. The membrane was then incubated with either anti-PKA $\text{C}\alpha$ or anti-PKA $\text{RII}\alpha$ at a concentration of 0.4 µg/ml (1:500) in TBS-T containing 10% (w/v) BSA for at least 5 h at room temperature, or overnight at 4°C , with maximum speed of agitation. Next, the membrane was washed for 6×10 min in TBS-T. Following the incubation of the membrane in a 1:10 000 dilution (in TBS-T plus 0.2% (w/v) BSA) of horseradish peroxidase (HRP)-conjugated goat antirabbit IgG for 1 h, the membrane was washed a further eight times for 10 min in TBS-T. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL).

To verify equal protein loading, blots were immersed in stripping buffer (6.25 mM Tris-HCl, pH 6.7, 2% (w/v) SDS and 0.7% (v/v) β -mercaptoethanol) and incubated for 30 min at 50°C . Stripped blots were then washed in TBS-T for 4×10 min, blocked and reprobed with anti-GAPDH antibody and HRP-conjugated goat antimouse IgG for 1 h and visualized as previously described. Densitometric quantification of immunoreactive bands was carried out with a BioRad Model GS-690 Imaging Densitometer with BioRad Molecular Analyst software, Version 1.4.

Statistical analysis

For secretion studies, treatments were carried out in quadruplicate with cells from an individual patient. Each experiment was repeated on at least three occasions with cells from three individual patients. Data obtained from each individual patient were internally referenced with the relevant experimental control standardized to 100% before being pooled with data from three individual patients and expressed as mean $\pm$ S.E.M. For immunoblotting studies, immunoblots shown are representative of experiments carried out with cells from three individual patients. Data obtained from densitometric measurements of the bands from three separate experiments have been pooled and are expressed as mean $\pm$ S.E.M. Statistical analysis was carried out on non-referenced data by one-way ANOVA with repeated measures, followed by Dunnett's multiple comparison test or unpaired *t*-test as appropriate, using the Graph Pad Prism software package (San Diego, CA, USA). Probabilities of less than or equal to 0.05 were accepted as significant.

Results

Basal progesterone secretion, cAMP accumulation and PKA $\text{C}\alpha$ and $\text{RII}\alpha$ expression during luteinization of human granulosa cells

Granulosa cells were cultured for 0–24 h (day 1), 24–48 h (day 2) and 48–72 h (day 3) in the absence of any

treatment. Basal progesterone secretion by cultured granulosa cells increased progressively over 3 days in culture with a significant increase being observed on day 3 of culture (Fig. 1A). During this transformation of granulosa cells to granulosa-lutein cells, basal cAMP levels remained unchanged (Fig. 1B). The expression of both PKA C α (Fig. 1C) and RII α (Fig. 1D) appeared to increase during culture when compared with expression on day 1; however, this increase was not statistically significant. Blots were stripped and probed with anti-GAPDH antibody to verify even protein loading (Fig. 1C).

Effect of hLH and forskolin and dibutyryl cAMP (dbcAMP) on progesterone production during luteinization

LH interaction with its G-protein-coupled receptor leads to the activation of the adenylate cyclase-cAMP-PKA signalling system. Luteinizing granulosa cells were treated with up to 1000 ng/ml hLH on days 1 and 3 of culture for 24 h. As expected, concentration-dependent stimulation of progesterone secretion was observed on each day of culture. The sensitivity of the cells to stimulation increased during luteinization; that is, on day 1 of culture 100 ng/ml hLH was the minimum dose required to elicit a significant increase in progesterone production (Fig. 2A), whereas 10 ng/ml hLH was the minimum dose required to elicit significant responses on day 3 of culture. Similar changes in sensitivity were obtained with a range of concentrations of the adenylate cyclase activator, forskolin (Fig. 2B), or the cell-permeable cAMP analogue, dbcAMP (Fig. 2C).

Effect of hLH and forskolin on cAMP accumulation during luteinization

For detailed dose-response studies, luteinizing granulosa cells were exposed to either hLH (0.1–1000 ng/ml) (Fig. 3A) or forskolin (0.001–10 μ M) (Fig. 3B) for 8 h on days 1 and 3 of culture. In addition, time-course studies were carried out for up to 8 h, using either 100 ng/ml hLH or 10 μ M forskolin (Fig. 4A–C). It is evident from the data presented in Fig. 3 that both hLH and forskolin were able to elicit dose-dependent increases in cAMP accumulation on each day of culture. Time-course studies revealed that on day 1, hLH had virtually no effect on cAMP accumulation relative to control (Fig. 4A). However, significant increases in cAMP production (relative to time-matched untreated control) were seen after 2-h incubation on day 2 (Fig. 4B) and after 10-min incubation on day 3 (Fig. 4C). Forskolin was able to elicit significant increases in cAMP production after 10-min incubation on day 1 and 5-min incubation on days 2 and 3 of culture, an effect that was maintained for up to 8 h. On day 3 of culture, there were no significant differences between hLH and forskolin in their relative abilities to stimulate cAMP accumulation.

Figure 1 Basal progesterone production, cAMP levels and PKA (C α /RII α) expression by luteinizing human granulosa cells. Granulosa cells were cultured for 24-h periods for up to 3 days, and spent media was analysed for (A) progesterone secretion, (B) cAMP levels, (C) GAPDH/C α expression and (D) RII α expression. Data are expressed as mean $\pm$ S.E.M. ($n=3$). * $P<0.05$ relative to day 1 of culture. Blots show data from a single experiment. Optical density data shown are cumulative and obtained from three experiments carried out with cells from three individual patients. Basal progesterone secretion on day 1 was 1.66 ± 0.5 nmol per 10^5 cells/24 h. Basal cAMP accumulation on day 1 was 22.3 ± 4.3 pmol/ 10^5 cells. Optical density values for GAPDH expression are expressed as mean percentage of day 1 $\pm$ S.E.M.: day 2, 96.8 ± 7.0 ; day 3, 104.1 ± 2.1 . $n=3$.

Figure 2 Effects of hLH, forskolin and dbcAMP on progesterone production. Granulosa cells were cultured for 24-h periods for up to 3 days, and on days 1 and 3, cells were treated with (A) hLH (0.1–1000 ng/ml), (B) forskolin (0.001–10 μ M) or (C) dbcAMP (0.5–5000 μ M). Spent medium was analysed for progesterone content. Data are expressed as mean $\pm$ S.E.M. $n=3$ experiments, using cells from three individual patients. * $P<0.05$; ** $P<0.01$ relative to control on the corresponding day of culture. Basal progesterone secretion on day 1 was 1.22 ± 0.7 nmol per 10^5 cells/24 h.

Figure 3 Effects of hLH and forskolin on cAMP generation. Granulosa cells were cultured for up to 3 days; on days 1 and 3 of culture, cells were incubated with either (A) hLH (0.1–1000 ng/ml) or (B) forskolin (0.001–10 μM) for 8 h. Spent medium was analysed for cAMP content. Data are expressed as mean $\pm$ S.E.M. $n=3$ experiments using cells from three individual patients. * $P<0.05$; ** $P<0.01$ relative to control on the corresponding day of culture. Basal cAMP accumulation (A) day 1: 35.2 ± 1.4 ; day 3: 32.1 ± 3.1 (B) day 1: 29.1 ± 2.3 ; day 3: 33.7 ± 1.1 pmol/ 10^5 cells.

Effect of hLH and forskolin on PKA α and RII α expression during luteinization

Given that gonadotrophins and other cAMP elevating agents were able to elicit concentration-dependent increases in progesterone synthesis during luteinization, we examined the effect of these agents on PKA α /RII α expression during luteinization. Surprisingly, exposure of cells to both hLH and forskolin markedly reduced both

PKA α and RII α subunit expression in a concentration-dependent manner on days 1 and 3 of culture. Thus, granulosa cells cultured in the presence of increasing doses of either hLH (Fig. 5A and B) or forskolin (Fig. 6A and B) expressed significantly less PKA α /RII α during each period of culture. Similar inhibitory responses were obtained with maximal doses of other physiological and pharmacological modulators of cAMP (PGE₂, dbcAMP and cholera toxin – data not shown).

Discussion

Following the LH surge and ovulation of an oocyte, the remaining granulosa and theca cells of the collapsed follicle undergo transition to luteal cells to form the corpus

luteum. In this study, granulosa cells cultured under basal conditions increased progesterone output as the cells became more luteinized. These observations are consistent with previous reports of granulosa cell luteinization (Wickings *et al.* 1986, Fisch *et al.* 1989, Fowkes *et al.* 2001). Interestingly, our present study shows that, as granulosa cells luteinized, their responsiveness to LH and other cAMP-elevating agents was changed in terms of progesterone secretion. Thus, on day 3 of culture, hLH was able to elicit a greater increase in progesterone secretion than that induced on day 1 of culture. Similar changes in responsiveness were noted with both forskolin and dbcAMP.

Changes in progesterone secretion induced by hLH and forskolin were accompanied by dose- and time-dependent increases in cAMP generation. However, despite the enhanced responsiveness of human granulosa cells to these agents, basal cAMP levels remained unchanged throughout culture. This stimulation of progesterone secretion by hLH is thought to be due to an increase in cAMP generation rather than an inhibition of phosphodiesterase activity (Marsh 1970), and it is known that increases in cAMP enhance steroidogenesis by increasing specific mRNAs, which encode components of the steroidogenic pathway, such as 3 β -HSD and Cytochrome P450 side-chain cleavage (Strauss *et al.* 1988).

The data presented here show that cAMP generation in luteinizing human granulosa cells was also found to be time-dependent. Notably, on day 1, hLH (100 ng/ml) could not significantly stimulate cAMP generation. This concentration of hLH (100 ng/ml) is supraphysiological. However, due to the nature of the cells (ovarian hyperstimulation) and the experimental design of this study (stimulation on day 1 of culture), the concentrations of LH used were effective throughout the culture period. Moreover, previous studies have used similar or greater doses of LH/hCG as an effective concentration to investigate progesterone secretion in this cell type (Polan *et al.* 1986, Khan-Dawood *et al.* 1989, Lee *et al.* 1997).

Exposure to an ovulatory dose of hCG can lead to a loss of response through a combination of receptor down-regulation (that is, a loss of receptor numbers) and desensitization, whereby the receptor becomes phosphorylated, resulting in its uncoupling from the Gs protein, and internalization (Ascoli *et al.* 2002). Thus, receptor

Figure 4 Time-dependent effects of hLH and forskolin on cAMP generation by luteinizing granulosa cells. Granulosa cells were incubated for up to 8 h in the presence or absence of either hLH (100 ng/ml) or forskolin (10 μ M) on (A) day 1, (B) day 2 and (C) day 3 of culture. Spent medium was analysed for cAMP content. Results are expressed as mean $\pm$ S.E.M. $n=3$ experiments using cells from three individual patients. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ relative to control at the corresponding time point. Basal cAMP values at time zero were 49.5 ± 2.4 on day 1, 45.3 ± 8.4 on day 2 and 55.3 ± 18.9 on day 3 pmol/ 10^5 cells.

Figure 5 Dose-dependent effects of hLH on PKA (C α /RII α) expression by luteinizing human granulosa cells. Luteinizing human granulosa cells were incubated in the presence or absence of increasing doses of hLH (10–1000 ng/ml) on days 1 and 3 of culture. After each 24-h incubation period, cells were lysed and 25 μ g protein were separated by SDS–PAGE, transferred to a PVDF membrane and probed with an antibody that recognized either the (A) C α or (B) RII α subunit of PKA. Blots shown represent data from a single experiment. Optical density data shown are cumulative and were obtained from three experiments carried out with cells from three individual patients. * $P < 0.05$; ** $P < 0.01$ relative to corresponding control.

Figure 6 Dose-dependent effects of forskolin on PKA (C α /RII α) expression by luteinizing human granulosa cells. Luteinizing human granulosa cells were incubated in the presence or absence of increasing doses of forskolin (0.1–10 μ M) on days 1 and 3 of culture. After each 24-h incubation period, cells were lysed, and 25 μ g protein were separated by SDS–PAGE, transferred to a PVDF membrane and probed with an antibody that recognized either the (A) C α or (B) RII α subunit of PKA. Blots shown represent data from a single experiment. Optical density data shown are cumulative and obtained from three experiments carried out with cells from three individual patients. * P <0.05; ** P <0.01 relative to corresponding control.

desensitization ultimately leads to a loss of any further signal (Leung & Steele 1992, Amsterdam *et al.* 2002). Receptor downregulation is a known mechanism by which target cells can reduce their response to an agonist through prolonged exposure (Amsterdam *et al.* 2002, Ascoli *et al.* 2002). Hence, a further challenge with LH, which utilizes the same receptor as hCG, would have little or no effect on progesterone secretion. In rat ovaries, for example, it has been shown that the preovulatory LH surge causes marked downregulation of the cell-surface LH receptor, which recovers upon luteinization (Segaloff *et al.* 1990, Peegel *et al.* 1994). The study by Peegel *et al.* (1994) lends support to our present data in terms of receptor downregulation with respect to cAMP generation, as, on day 1 of culture, 100 ng/ml hLH were unable to stimulate cAMP generation, whereas, on day 3 of culture, hLH significantly increased cAMP generation compared with the control. However, forskolin was able to elicit increases in cAMP generation after 10 min on each day of culture. This response could be explained by the fact that forskolin acts in a receptor-independent manner by stimulating adenylate cyclase in order to raise intracellular cAMP (Jammes *et al.* 1988, Asboth *et al.* 2001).

Here we present data to show that chronic elevation of cAMP, whether by physiological (e.g. hLH) or pharmacological (e.g. dbcAMP and forskolin) means, leads to an increase in progesterone production coincident with a decrease in both the $C\alpha$ and regulatory $RII\alpha$ subunits of PKA, during luteinization of human granulosa cells. As progesterone output increases despite an apparent decrease in $C\alpha$ expression, it is possible that this decrease is due to the raised steroidal output. However, similar findings were reported in non-steroidogenic tissue where prolonged exposure of rat hepatocytes to glucagon was found to lead to prolonged, enhanced cAMP levels, which were associated with a decrease in $C\alpha$ expression that could not be accounted for by changes in $C\alpha$ mRNA expression (Houge *et al.* 1990). Similar results have also been reported in rat pituitary GH3 cells (Richardson *et al.* 1990) and in mutant lymphocytes (Steinberg & Agard 1981), where the apparent decrease in the expression of $C\alpha/RII\alpha$ subunit was suggested to have been due to an enhanced rate of degradation. Hence, increased degradation of $C\alpha/RII\alpha$ subunits in response to prolonged exposure to cAMP provides the most likely explanation for the decreases in PKA subunit expression observed in the present study.

It has been generally accepted that LH activates its G-protein-coupled receptor in the ovary, leading to the activation of adenylate cyclase, and resulting in an increase in intracellular cAMP, and thus an increase in the activity of PKA, that ultimately increases progesterone secretion. However, the apparent decreases in the expression of PKA subunits induced by LH (and other agents that raise intracellular cAMP levels), as demonstrated in this study, suggest that the ability of luteinizing granulosa cells to secrete progesterone can be dissociated from increases in

PKA $C\alpha$ and $RII\alpha$ expression; that is, steroid synthesis becomes less dependent on PKA $C\alpha$ and $RII\alpha$ expression as luteinization proceeds. While changes in PKA activity were not determined in this study, the presumption is that the decrease in expression of the PKA subunits ($C\alpha$ and $RII\alpha$) did indeed lead to reduction in PKA activity.

These results thus suggest that an increase in PKA $C\alpha$ and $RII\alpha$ expression/activity may not be the sole means by which progesterone secretion is regulated during luteinization. In man, the functional lifespan of the CL is approximately 14–16 days, during which time progesterone secretion is maintained under the influence of LH (Hutchinson & Zeleznik 1984, Hall 1993). Therefore, extrapolation of our current findings that LH treatment *in vitro* caused a profound inhibition ($42.6 \pm 4.8\%$) of $C\alpha$ expression suggests that during luteinization and CL development *in vivo*, PKA expression/activity should decrease. This is consistent with the reported inhibitory effects of treatment *in vivo* with hCG on PKA activity in the macaque CL (Benyo & Zeleznik 1997). Hence, despite the decrease in expression of both the $RII\alpha$ and $C\alpha$ subunits of PKA, the cells continue to secrete large amounts of progesterone. This notion implies that other signalling pathways may be involved in the regulation of progesterone secretion by luteinizing granulosa cells.

The discovery of a new class of cAMP-binding proteins, the cAMP-guanine nucleotide exchange factors (cAMP-GEFs) (de Rooij *et al.* 1998), has yielded an alternative intracellular target to PKA that could be activated by cAMP. Although transcripts for both cAMP-GEFI and cAMP-GEFII have been identified in the rat ovary (Gonzalez-Robayna *et al.* 2000), expression has not yet been identified at the protein level in the human ovary. Thus, in the accompanying paper, we provide evidence to support the notion that cAMP-GEFI signalling contributes to the regulation of progesterone secretion during luteinization.

Acknowledgements

We are grateful to the patients and staff at Lister Hospital for the provision of follicular aspirates. This work was supported by the BBSRC (99/B1/S/05251) and the Wellcome Trust (056630/Z/99/JMW/NP/JF).

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Received in final form 27 May 2004

Accepted 30 June 2004

Made available online as an

Accepted Preprint 13 July 2004