Progesterone secretion by luteinizing human granulosa cells: a possible cAMP-dependent but PKA-independent mechanism involved in its regulation

E C Chin and D R E Abayasekara

Reproduction and Development Group, Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK
(Requests for offprints should be addressed to E C Chin; Email: echin@rvc.ac.uk)

Abstract

The corpus luteum formed after luteinization of follicular cells secretes progesterone under the control of luteinizing hormone (LH). Binding of LH to its G-protein-coupled receptor leads to the activation of the adenylate cyclase/cyclic AMP (cAMP)/cAMP-dependent protein kinase (PKA) signalling pathway. The identification of a new class of cAMP-binding proteins termed 'guanine nucleotide exchange factors' (cAMP-GEFs) provides a means by which changes in cAMP could yield actions that are independent of PKA. Hence, in this study, we have explored the hypothesis that steroidogenesis in luteinizing cells is mediated in both a cAMP/PKA-dependent and cAMP-dependent, but PKA-independent, manner. Human granulosa cells were isolated from follicular aspirates of women undergoing assisted conception. Luteinizing human granulosa cells were cultured for up to 3 days in the presence of human (h)LH and the adenylate cyclase activator forskolin in the added presence or absence of increasing doses of the PKA inhibitors H89 (N-[2-(4-bromocinnamylamino)ethyl] 5-isoquinoline) and PKI (myristoylated protein kinase A inhibitor amide 14–22) or the cAMP antagonist, Rp-cAMP. Agonist-stimulated progesterone secretion was inhibited in a dose-dependent manner by the PKA inhibitors and the cAMP antagonist, with decreasing sensitivity as luteinization progressed. Pretreatment of granulosa cells for 4 h with human (h)LH

reduced the effectiveness of H89 in inhibiting progesterone secretion. Under basal conditions, cAMP-GEFI expression increased progressively throughout culture, and this could be further enhanced when cells were incubated with increasing doses of LH and forskolin. Furthermore, incubation of cells in the presence of increasing concentrations of the novel cAMP-GEF-specific cAMP analogue, 8 CPT-2 ME-cAMP (8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate), increased progesterone secretion in a dose-dependent manner. The results show that increases in cAMP generated by LH and forskolin, in addition to activating PKA, also induce increases in cAMP-GEFI protein expression in luteinizing human granulosa cells. In addition, activation of cAMP-GEFI results in increased progesterone secretion. Hence, increases in cAMP lead to the activation of PKA-dependent, as well as PKA-independent but cAMP-dependent (via cAMP-GEFI), signalling mechanisms. Since cAMP-GEFs have the capacity to activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) signalling pathways, these may provide the potential mechanisms by which cAMP-dependent but PKA-independent progesterone synthesis is regulated.

Journal of Endocrinology (2004) **183**, 51–60

Introduction

It is well established that binding of luteinizing hormone (LH) in the ovary to its G protein-coupled receptor leads to the generation of intracellular cyclic AMP (cAMP) and the activation of cAMP-dependent protein kinase (PKA) (Marsh & Savard 1966, Marsh 1970, Leung & Steele 1992). Although the activation of the cAMP/PKA signalling pathway is the major signalling pathway involved in the regulation of ovarian steroidogenesis, binding of LH to its receptor can also increase phosphatidyl inositol (PI) turnover and elevate intracellular calcium (Gilchrist

et al. 1996, Hirsch *et al.* 1996), hence activating PKA-independent signalling pathways such as the phospholipase C/protein kinase C and the phospholipase A₂/arachidonic acid signalling pathways (Guderman *et al.* 1992, Cooke 1999).

Until recently, PKA was thought to be the only intracellular target of cAMP. In 1998, a new class of cAMP-binding proteins was discovered (de Rooij *et al.* 1998, Kawasaki *et al.* 1998). These proteins, termed 'cAMP guanine nucleotide exchange factors' (cAMP-GEFs) and also known as Epacs (exchange proteins directly activated by cAMP), represent an alternative target to PKA that can

be activated by cAMP. cAMP-GEFs contain a cAMP-binding domain with significant sequence homology to both R subunits of PKA, and a guanine nucleotide exchange factor (GEF) domain which functions to exchange GTP for GDP (de Rooj *et al.* 1998, Kawasaki *et al.* 1998). cAMP-GEFs were first shown to be an exchange factor for the small GTPase, Rap 1 and, more recently, Rap 2 (de Rooj *et al.* 2000). GTP-bound Rap 1 leads to the activation and initiation of a cascade of protein kinases of the mitogen-activated protein kinase (MAPK) signal transduction pathway.

The activation of the MAPK signal transduction pathway has been implicated in the regulation of steroidogenesis. Studies performed in rat (Das *et al.* 1996) and pig (Cameron *et al.* 1996) granulosa cells demonstrated that gonadotrophin stimulation led to the activation of extracellular signal-related kinase 1/2 (ERK1/2), a member of the MAPK family, in a cAMP/PKA-dependent manner. More recently, Dewi *et al.* (2002) carried out a study to determine the effects of LH, human chorionic gonadotropin (hCG) and other agents that elevate cAMP on ERK1/2 in human granulosa-lutein cells. The results showed that agents that elevate cAMP activated ERK1/2 in a dose- and time-dependent manner, a response that could be reduced by preincubation with PKA inhibitors. MAPK inhibitors reduced gonadotrophin-induced ERK1/2 activation but had no effect on gonadotrophin-induced progesterone secretion. However, ERK1/2 activation induced by pharmacological agents that elevate cAMP (cholera toxin, forskolin and dibutyryl cAMP) was blocked by MAPK inhibitors coincident with a decrease in progesterone production. Thus, ERK1/2 activation is involved in the regulation of progesterone secretion by human granulosa-lutein cells and is stimulus dependent. However, the nature of the cAMP-dependence of ERK1/2 activation remains unelucidated. cAMP-GEFI is an intermediate through which the two pathways could be linked.

We have previously shown that, during luteinization of human granulosa cells, gonadotrophin-induced progesterone secretion is coincident with a decrease in the expression of the active catalytic subunit of PKA (Chin *et al.* 2004), a finding which suggests that, during luteinization, increases in progesterone secretion can be dissociated from increases in PKA expression/activity; that is, steroid synthesis becomes less dependent upon PKA as luteinization proceeds. Both cAMP-GEFs (I and II) and the effector Rap are expressed in granulosa cells at the level of mRNA (Gonzalez-Robayna *et al.* 2000). Since gonadotrophins can induce activation of the MAPK signal transduction pathway (Cameron *et al.* 1996, Das *et al.* 1996, Dewi *et al.* 2002), it is conceivable that changes in cAMP-GEF expression/activity may play a role in gonadotrophin-induced MAPK activation and/or progesterone secretion in granulosa cells. Hence, the aims of this study were to investigate changes in cAMP-GEFI expression as human

granulosa cells luteinize under basal conditions and in response to gonadotrophin stimulation in relation to progesterone secretion.

Materials and Methods

Materials

Human LH (hLH; NIDDK-hLH-B-SIAFP-2 – 6100 IU/mg) was kindly donated by A F 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). [³H]Progesterone, ¹²⁵I-cAMP-TME and Hyperfilm ECL were purchased from Amersham. Protogel and SDS tank buffer were purchased from National Diagnostics (Hull, UK). Goat polyclonal anti-cAMP-GEFI was purchased from Santa Cruz (Calne, UK). Mouse monoclonal antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Biogenesis (Poole, UK). 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8 CPT-2 ME-cAMP) was purchased from Biolog Life Science Institute (Bremen, Germany). PKI (myristoylated protein kinase A inhibitor amide 14–22) was purchased from Calbiochem (Nottingham, UK). Rabbit anti-goat IgG and bichinchoninic acid (BCA) reagent were purchased from Pierce (Chester, UK). H89 (N-[2-(4-bromocinnamylamino) ethyl] 5-isoquinoline), IgG, and 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 from 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 the subcutaneous administration of a GnRH analogue (Suprecur; Shire Pharmaceuticals, Andover, UK; 500 µg/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; 5000–10000 IU) 36 h before 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.* 1993).

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, 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. Cells were counted 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

Granulosa cells were plated in 35 mm culture dishes or in 6-well plates at a density of 1×10^6 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% O₂ and 5% CO₂.

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 Na₂HPO₄·2H₂O, 80 mM Na₂HPO₄·12H₂O, 150 mM NaCl, 1% (w/v) NaN₃ and 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.

Preparation of luteinizing human granulosa cell lysates

After treatment of granulosa cells as described in Figure legends, 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 AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride] and 50 µg/ml leupeptin) was added to the cells, which were then left on ice for 10 min. The luteinizing granulosa cells were then scraped from the culture dish into 1.5 ml microfuge tubes. Cell lysates were then boiled at 100 °C for 5 min. After determination of protein content by the BCA method, 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 rpm for 1 min prior to electrophoresis.

SDS–PAGE and Western immunoblotting

Proteins of interest within whole granulosa-cell lysates were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Wheeler-Jones *et al.* 1996). After electrophoresis, proteins were transferred to an Immobilon-polyvinylidene difluoride (PVDF) membrane, using the Bio-Rad 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 and 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 anti-cAMP-GEFI (1:500 dilution) 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. The membrane was then washed for 610 min in TBS–T. After 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 rabbit antigoat 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 410 min, blocked and reprobed with anti-GAPDH antibody and appropriate secondary antibody, 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, treatment was carried out in quadruplicate with cells from an individual patient. Each individual experiment was repeated on at least three occasions with cells obtained from three separate 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 three individual 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 a Graph Pad Prism software package (San Diego, CA, USA). Probabilities of less than or equal to 0.05 were accepted as significant.

Results

Effect of PKA inhibition by H89 on hLH-, forskolin- and dbcAMP-induced progesterone secretion during luteinization of human granulosa cells

Since we have previously shown that gonadotrophins, as well as other agents that raise intracellular cAMP, were able to increase progesterone secretion during the luteinization of human granulosa cells (Chin *et al.* 2004), we went on to investigate the effect of PKA inhibition on agonist-induced progesterone secretion. Granulosa cells were incubated on days 1 (0–24 h) and 3 (48–72 h) of culture in the presence of hLH (100 ng/ml), the adenylate cyclase activator forskolin (10 μ M) or the cell-permeable cAMP analogue, dbcAMP (5000 μ M), with or without increasing doses of the PKA inhibitor, H89 (0.1–10 μ M). As can be seen in Fig. 1A–C, agonist-induced progesterone secretion was inhibited in a dose-dependent manner on each day of culture. Notably, granulosa cells became less susceptible to inhibition as luteinization progressed. Thus, hLH-induced progesterone secretion was significantly inhibited with 1 μ M H89 on day 1 of culture, but this dose could not significantly inhibit hLH-induced progesterone secretion on day 3 of culture (Fig. 1A). This changing susceptibility to inhibition by H89 was also observed when either forskolin (Fig. 1B) or dbcAMP (Fig. 1C) was used to stimulate progesterone secretion. Similar patterns of response were obtained with the structurally dissimilar inhibitor of PKA, myr-PKI_{14–22} (Fig. 2A), as well as the cAMP antagonist, Rp-cAMP (Fig. 2B).

Basal expression of cAMP-GEFI and progesterone secretion during luteinization of human granulosa cells

Given that granulosa cells become less susceptible to inhibition as luteinization progressed, human granulosa cells were cultured for up to 3 days under basal conditions. After the removal of culture medium for the analysis of progesterone content by RIA, cells were lysed, and cAMP-GEFI expression in these lysates was determined by SDS–PAGE followed by Western blotting. Under basal conditions, the expression of cAMP-GEFI was found to increase significantly as the granulosa cells luteinized during the 3-day period of culture, and equal protein loading was verified by blots being stripped and reprobed with anti-GAPDH antibody (Fig. 3A). Increases in cAMP-GEFI expression were coincident with a significant increase in progesterone secretion (Fig. 3B).

Effect of hLH on the expression of cAMP-GEFI and progesterone secretion during luteinization of human granulosa cells

Human granulosa cells were treated with increasing doses of hLH (10–1000 ng/ml) for 24 h on days 1 and 3 of culture. Culture medium was removed and analysed for progesterone content. After the removal of the culture medium, cells were lysed and analysed for cAMP-GEFI expression by SDS–PAGE followed by Western blotting (Fig. 4A). Thus, the data in Fig. 4 show that treatment of cells with hLH significantly increased cAMP-GEFI expression in a dose-dependent manner on each day of culture. Similar patterns of expression were observed when cells were treated with the adenylate cyclase activator, forskolin (data not shown). The increase in cAMP-GEFI expression was coincident with a dose-dependent increase in progesterone secretion (Fig. 4B).

Effect of a cAMP-GEF-specific cAMP analogue, 8CPT-2Me-cAMP, on progesterone secretion by luteinizing human granulosa cells

Recently, a novel cAMP-GEF-specific cAMP analogue, 8 CPT-2 Me-cAMP, has been developed which effectively discriminates between the cAMP-GEF and PKA signalling pathways (Enserink *et al.* 2002). Hence, to determine whether the cAMP-GEF signalling pathway was involved in progesterone secretion, on days 1 and 3 of culture, luteinizing human granulosa cells were cultured in the presence or absence of increasing doses of 8 CPT-2 Me-cAMP (0.1–100 μ M). Interestingly, this cAMP-GEF-specific cAMP analogue was able to elicit dose-dependent increases in progesterone secretion on each day of culture (Fig. 5).

Discussion

It is well documented that the cAMP/PKA signalling pathway is the major signalling pathway involved in the regulation of progesterone secretion. However, data presented in this study suggest that, during luteinization, progesterone secretion becomes less dependent on the activity of PKA. We have shown that the concentration of H89 and PKI (Harris *et al.* 1997) required to inhibit progesterone synthesis is greater on day 3 than day 1. Similar findings were seen with the cAMP antagonist Rp-cAMP, a finding which is not surprising since cAMP is an absolute requirement for the activation of PKA.

Inhibiting PKA with H89 effectively inhibits progesterone secretion during luteinization, in agreement with studies using rat granulosa cells (Morris & Richards 1995). However, the present data consistently showed that the concentration of inhibitor required to inhibit progesterone synthesis was greater on day 3 than on day 1. Data presented in our previous study showed that basal PKA Ca

Figure 1 Dose-dependent effects of H89 on hLH-, forskolin- and dbcAMP-stimulated progesterone secretion by luteinizing granulosa cells. Granulosa cells were cultured for up to 3 days, and on days 1 and 3, cells were incubated in the presence or absence of hLH (100 ng/ml) (A), forskolin (10 μ M) (B) or dbcAMP (5000 μ M) (C), in addition to increasing doses of H89 (0.1–100 μ M) for 24 h. 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 values for progesterone: 1.06 ± 0.2 (day 1) and 2.37 ± 1.3 (day 3) nmol/ 10^5 cells per 24 h.

Figure 2 Dose-dependent effects of PKI (A) and (B) Rp-cAMP on hLH-stimulated progesterone secretion by luteinizing granulosa cells. Granulosa cells were cultured in the presence or absence of hLH (100 ng/ml) in addition to increasing doses of Rp-cAMP (0.25–1 mM) on days 1 and 3 of culture for 24 h. Spent medium was assessed for progesterone content by RIA. 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 values: 1.23 ± 0.4 (day 1) and 2.15 ± 0.9 (day 3) nmol/ 10^5 cells per 24 h.

expression appeared to increase throughout the 3-day culture period, although not significantly (significance might have been achieved with a larger sample number). Thus, following inhibition by hLH, the absolute quantity of Ca that remained on day 3 was greater than that on day 1. Thus, it is not surprising that on day 3 more H89

was required to inhibit progesterone secretion than that required on day 1, consistently with the view that, during luteinization, progesterone secretion becomes less dependent on the expression/activity of PKA.

Transcripts for both cAMP-GEFI and cAMP-GEFII have been identified in rat granulosa cells, and the results

Figure 3 Basal expression of cAMP-GEFI and progesterone secretion during luteinization of human granulosa cells. Granulosa cells were cultured under basal conditions for up to 3 days. Culture medium was removed on each day, and lysed or cultured for 24-h periods for up to 3 days. (B) After each 24-h incubation period, culture medium was removed and analysed for progesterone content. (A) Cells were lysed, and 50 μ g protein were separated by SDS-PAGE, transferred to a PVDF membrane and probed with an antibody that recognized cAMP-GEFI. The blot shown is of a single representative experiment. Optical density data shown are cumulative and obtained from three experiments with cells from three individual patients. ** $P < 0.01$ relative to day 1. Optical density values for GAPDH expression are expressed as mean percentage of day 1 $\pm$ s.e.m.: day 2, 102.8 ± 5.1 ; day 3, 99.1 ± 2.1 . ($n=3$). Progesterone secretion on day 1 was 1.38 ± 0.4 nmol/ 10^5 cells per 24 h.

showed that transcripts were not hormonally regulated, although protein expression was not determined (Gonzalez-Robayna *et al.* 2000). Data presented herein show for the first time that cAMP-GEFI protein is

expressed in luteinizing human granulosa cells and that expression increases under basal conditions coincident with increased progesterone secretion during luteinization of human granulosa cells. In addition, cAMP-GEFI

Figure 4 Effect of hLH on the expression of cAMP-GEFI and progesterone secretion during luteinization of 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 (A) 50 µg protein were separated by SDS–PAGE, transferred to a PVDF membrane and probed with an antibody that recognized cAMP-GEFI. (B) Spent medium was removed and assessed for progesterone content. The blots shown represent data from a single experiment. Optical density data shown are cumulative and were obtained from three experiments with cells from three individual patients. ** $P < 0.01$ relative to the control. Basal progesterone secretion on day 1 was 1.32 ± 0.07 nmol/ 10^5 cells per 24 h.

protein expression can be increased in a dose-dependent manner in response to hLH and the adenylate cyclase activator forskolin.

A novel cAMP-GEF specific cAMP analogue, 8 CPT-2 Me-cAMP, has been designed which discriminates between cAMP-GEF and PKA. Specificity has been

Figure 5 Effects of the cAMP-GEF-specific cAMP analogue, 8 CPT-2 Me-cAMP, on progesterone secretion by luteinizing human granulosa cells. Granulosa cells were cultured in the presence or absence of 8 CPT-2 Me-cAMP (0.1–100 μM) on days 1 and 3 of culture for 24 h. Spent medium was assessed for progesterone content by RIA. Data are expressed as mean ± 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 values for day 1 were 1.15 ± 0.02 nmol/ 10^5 cells per 24 h.

demonstrated by studies which show that this cAMP analogue specifically activates cAMP-GEFI in NIH3T3-A14-Epac 1 cells, as illustrated by the induction of Rap 1 activation without inducing phosphorylation of cAMP response element binding protein (CREB) (Enserink *et al.* 2002). This compound has recently been used to discriminate between cAMP-dependence and PKA-dependence in pancreatic beta cells (Kang *et al.* 2003). Treatment of luteinizing granulosa cells with this cAMP-GEF-specific cAMP analogue dose-dependently increased progesterone secretion on each day of culture. Thus, activation of cAMP-GEFI can also increase progesterone secretion in luteinizing human granulosa cells. Given that activation of GEFs has the potential to activate the MAPK signalling cascade, LH-induced progesterone secretion may also involve the activation of MAPK through the activation of cAMP-GEFI.

There are conflicting reports on the effects of ERK on gonadotrophin-induced steroidogenesis. Seger *et al.* (2001) demonstrated in granulosa cell-derived cell lines that gonadotrophin induced ERK activation as well as progesterone secretion. However, this study showed that inhibition of ERK increased gonadotrophin-induced progesterone secretion. Conversely, Gyles *et al.* (2001) demonstrated in Y1 and MA-10 cells that ERK inhibition decreased forskolin-induced steroid secretion. More recently, Dewi *et al.* (2002) demonstrated in human granulosa-luteal cells that gonadotrophins and agents that elevate intracellular cAMP levels induce time- and dose-dependent activation of ERK. This activation was found to be partially PKA dependent, suggesting that

ERK activation also involves a PKA-independent component, which may be mediated through the activation of cAMP-GEFs. Thus, it is conceivable that changes in cAMP-GEF expression/activity may play a role in gonadotrophin-induced MAPK activation and/or progesterone secretion in luteinizing human granulosa cells. The effectors Rap 1 and Rap 2, as well as Raf-1 and B-Raf, are expressed in rat granulosa cells (Gonzalez-Robayna *et al.* 2000). Gonadotrophin-induced activation of phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) signalling pathways in granulosa cells has been shown to be PKA-independent (Gonzalez-Robayna *et al.* 2000, Richards, 2001). The recent finding that PI3-K inhibitors inhibited progesterone synthesis (Zelevnik *et al.* 2003) lends support to the notion that activation of PI3-K, via cAMP-GEFs, may provide a cAMP-dependent but PKA-independent mechanism through which progesterone synthesis could be regulated in luteinized granulosa cells.

In summary, the regulation of chronic progesterone secretion may now be seen as less dependent on the expression/activity of PKA. cAMP-GEFI protein expression has been demonstrated in luteinizing human granulosa cells for the first time, and this increased over the 3-day culture period, and could be further enhanced in response to hLH and the adenylate activator forskolin. In addition, specific activation of cAMP-GEFI resulted in an increase in progesterone secretion. Thus, chronic progesterone secretion by luteinizing human granulosa cells may be regulated by PKA-independent mechanisms that involve the activation of cAMP-GEFI.

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).

References

- Abayasekara DRE, Jones PM, Persaud SJ, Michael AE & Flint APF 1993 Prostaglandin $F_{2\alpha}$ activates protein kinase C in human ovarian cells. *Molecular and Cellular Endocrinology* **91** 51–57.
- Aldred LF & Cooke B 1983 The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells, using an NADH exclusion test for determination of viability. *Journal of Steroid Biochemistry* **18** 411–414.
- Cameron MR, Foster JS, Bukovsky A & Wimalasena J 1996 Activation of MAPK by gonadotrophins and cyclic adenosine 3',5'-monophosphates in porcine granulosa cells. *Biology of Reproduction* **55** 111–119.
- Chin EC, Harris TE & Abayasekara DRE 2004 Changes in cAMP-dependent protein kinase (PKA) and progesterone secretion in luteinizing human granulosa cells. *Journal of Endocrinology* (Submitted).
- Cooke BA 1999 Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Molecular and Cellular Endocrinology* **151** 25–35.
- Das S, Maziels ET, DeManno D, St Clair E, Adam SA & Hunzicker-Dunn M 1996 A stimulatory role of cyclic adenosine 3',5'-monophosphate in FSH activates MAPK signaling pathway in rat ovarian granulosa cells. *Endocrinology* **137** 967–974.
- Dewi DA, Abayasekara DRE & Wheeler-Jones CPD 2002 Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa-lutein cells is stimulus specific. *Endocrinology* **143** 877–888.
- Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL & Bos JL 2002 A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nature Cell Biology* **4** 901–906.
- Gilchrist RL, Ryu KS, Ji I & Ji TH 1996 The luteinizing hormone/chorionic gonadotropin receptor has distinct transmembrane conductors for cAMP and inositol phosphate signals. *Journal of Biological Chemistry* **271** 19283–19287.
- Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL & Richard JS 2000 Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for a kinase-independent signalling by FSH in granulosa cells. *Molecular Endocrinology* **14** 1283–1300.
- Guddermand T, Birnbaumer M & Birnbaumer I 1992 Evidence for dual coupling of the murine luteinizing hormone receptor to adenylate cyclase and phosphoinositide breakdown and Ca^{2+} mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *Journal of Biological Chemistry* **267** 4479–4488.
- Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ & Jones PM 2001 ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. *Journal of Biological Chemistry* **276** 34888–34895.
- Harris TE, Persaud SJ & Jones PM 1997 Pseudosubstrate inhibition of cAMP-dependent protein kinase in intact pancreatic islets: effects on cyclic AMP-dependent and glucose-dependent insulin secretion. *Biochemistry and Biophysics Research Communications* **232** 648–651.
- Hirsch B, Kudo M, Naro F, Conti M & Hsueh AJ 1996 The C-terminal third of the human luteinizing hormone (LH) receptor is important for inositol phosphate release: analysis using chimeric human LH/follicle-stimulating hormone receptors. *Molecular Endocrinology* **10** 1127–1137.
- Kang G, Joseph JW, Chepurmy OG, Monaco M, Wheeler MB, Bos JL, Schwede F, Genieser HG & Holz GG 2003 Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca^{2+} induced Ca^{2+} release and exocytosis in pancreatic beta-cells. *Journal of Biological Chemistry* **278** 8279–8285.
- Kawasaki H, Springett GM, Mochizuki N, Nakaya M, Matsuda M, Housman DE & Graybiel AM 1998 A family of cAMP-binding proteins that directly activate Rap1. *Science* **282** 2275–2279.
- Leung PC & Steele GL 1992 Intracellular signalling in the gonads. *Endocrine Reviews* **13** 476–498.
- Marsh JM 1970 The stimulatory effect of luteinizing hormone on adenylate cyclase in the bovine corpus luteum. *Journal of Biological Chemistry* **245** 1596–1603.
- Marsh JM & Savard K 1966 The stimulation of progesterone synthesis in bovine corpora lutea by cAMP. *Steroids* **8** 133–148.
- Morris JK & Richards JS 1995 Luteinizing hormone induces prostaglandin endoperoxide synthase-2 and luteinization in vitro by A-kinase and C-kinase pathways. *Endocrinology* **136** 1549–1558.
- Pallikaros Z, Schulster D, Baldwin SA, Helliwell RJ, Michael AE & Cooke BA 1995 Characterization of site-directed antibodies to the LH receptor in functionally active gonadal cells and their differential effects on LH-stimulated signal transduction in Leydig tumour (MA-10) cells. *Molecular and Cellular Endocrinology* **14** 57–68.
- Richards JS 2001 New signalling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Molecular Endocrinology* **15** 209–218.
- Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF III & Amsterdam A 2001 The ERK signalling cascade inhibits gonadotrophin-stimulated steroidogenesis. *Journal of Biological Chemistry* **276** 13957–13964.
- de Rooij J, Zwartkruis FJT, Verheijen MHG, Cool RH, Nijman SMB, Wittinghofer A & Bos JL 1998 Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cAMP. *Nature* **396** 474–477.
- de Rooij J, Rehmann H, van Treist M, Cool RH, Wittinghofer A & Bos JL 2000 Mechanisms of regulation of the Epac family of cAMP-dependent RapGEFs. *Journal of Biological Chemistry* **275** 20829–20836.
- Wheley GE, Luck MR & Hearn JP 1988 Stimulation of cultured human granulosa cells with melatonin and catecholamines. *Journal of Reproduction and Fertility* **84** 669–677.
- Wheeler-Jones CPD, May MJ, Houlston RA & Pearson JD 1996 Inhibition of MAP kinase kinase (MEK) blocks endothelial PGI₂ release but has no effect on von Willebrand factor secretion or E-selectin expression. *FEBS Letters* **388** 180–184.
- Zeleznik AJ, Saxena D & Little-Ihrig L 2003 Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. *Endocrinology* **144** 3985–3994.

Received in final form 27 May 2004

Accepted 30 June 2004

Made available online as an

Accepted Preprint 13 July 2004