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

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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-GEF 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′-0-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-GEF protein expression in luteinizing human granulosa cells. In addition, activation of cAMP-GEF results in increased progesterone secretion.

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 Rooy 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 Rooy 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. Protigel 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 antialglyceralddehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Biogenesis (Poole, UK). 8-(4-Chlorophenylthio)-2′,0-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-isouquinoline), 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 Δ5–3β-hydroxysteroid dehydrogenase: Δ5–4–isomerase (3βHSD) 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 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 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 ± 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 ± S.E.M. Statistical analysis was carried out on non-referenced data by one-way ANOVA with repeated measures followed by Dunnet’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 µM) or the cell-permeable cAMP analogue, dbcAMP (5000 µM), with or without increasing doses of the PKA inhibitor, H89 (0.1–10 µM). 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 µM 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-PKI14–22 (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, 8CPT-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 (0.1–1000 µM). 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
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 μM) (B) or dbcAMP (5000 μM) (C), in addition to increasing doses of H89 (0·1–100 μM) for 24 h. Spent medium was analysed for progesterone content. 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 values for progesterone: 1·06 ± 0·2 (day 1) and 2·37 ± 1·3 (day 3) nmol/10^5 cells per 24 h.

Figure 1
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 Cα 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-GEF I and cAMP-GEF II have been identified in rat granulosa cells, and the results

![Figure 2](https://www.endocrinology-journals.org)
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-GEF I 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-GEF I and progesterone secretion...
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 4 Effect of hLH on the expression of cAMP-GEF I 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-GEF I. (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.
demonstrated by studies which show that this cAMP analogue specifically activates cAMP-GEF I 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-GEF I 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-GEF I.

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. 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 (Zeleznik 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-GEF I 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-GEF I 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-GEF I.
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References


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