Development 136, 1869-1878 (2009) doi:10.1242/dev.035238

Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte

Rachael P. Norris[1], William J. Ratzan[1], Marina Freudzon[1], Lisa M. Mehlmann[1], Judith Krall[2], Matthew A. Movsesian[2], Huanchen Wang[3], Hengming Ke[3], Viacheslav O. Nikolaev[4,*] and Laurinda A. Jaffe[1,*]

Mammalian oocytes are arrested in meiotic prophase by an inhibitory signal from the surrounding somatic cells in the ovarian follicle. In response to luteinizing hormone (LH), which binds to receptors on the somatic cells, the oocyte proceeds to second metaphase, where it can be fertilized. Here we investigate how the somatic cells regulate the prophase-to-metaphase transition in the oocyte, and show that the inhibitory signal from the somatic cells is cGMP. Using FRET-based cyclic nucleotide sensors in follicle-enclosed mouse oocytes, we find that cGMP passes through gap junctions into the oocyte, where it inhibits the hydrolysis of cAMP by the phosphodiesterase PDE3A. This inhibition maintains a high concentration of cAMP and thus blocks meiotic progression. LH reverses the inhibitory signal by lowering cGMP levels in the somatic cells (from ~2 μM to ~80 nM at 1 hour after LH stimulation) and by closing gap junctions between the somatic cells. The resulting decrease in oocyte cGMP (from ~1 μM to ~40 nM) relieves the inhibition of PDE3A, increasing its activity by ~5-fold. This causes a decrease in oocyte cAMP (from ~700 nM to ~140 nM), leading to the resumption of meiosis.

KEY WORDS: cAMP, cGMP, Gap junctions, Meiosis, Mouse ovarian follicle, Oocyte

INTRODUCTION

Fully grown mammalian oocytes are held in meiotic prophase arrest by a signal from the surrounding somatic cells of the ovarian follicle. The outer layers of somatic cells (mural granulosa), rather than the inner cumulus cells that directly surround the oocyte, are considered to be the primary source of the meiosis-inhibitory signal, as removal of oocytes or cumulus-oocyte complexes from the follicle causes meiosis to resume (Pincus and Enzmann, 1935). The mural granulosa cells are also the site of action of luteinizing hormone (LH), which is the physiological stimulus for the prophase-to-metaphase transition (Eppig et al., 2004; Mehlmann, 2005a; Jones, 2008).

Maintenance of prophase arrest also requires elevated cAMP levels in the oocyte (Cho et al., 1974; Masciarelli et al., 2004). The oocyte generates cAMP by way of a constitutively active heterotrimeric G protein (G_s)-linked receptor, GPR3 or GPR12, which acts to stimulate adenylyl cyclase (Mehlmann et al., 2002; Mehlmann et al., 2004; Horner et al., 2003; Freudzon et al., 2005; Hinckley et al., 2005; Ledent et al., 2005; Mehlmann, 2005b). cAMP maintains meiotic prophase arrest by way of protein kinase A (PKA)-mediated phosphorylation of proteins that regulate cyclin-dependent protein kinase (Jones, 2008).

cAMP levels in the oocyte decrease with time after its removal from the follicle (Vivarelli et al., 1983; Törnell et al., 1990a), indicating that the somatic cells contribute to maintaining elevated cAMP in the oocyte. Isolation of the oocyte does not decrease G_s activation by GPR3, indicating that the meiosis-inhibitory signal is not a GPR3 agonist (Freudzon et al., 2005). Instead, the signal appears to act by way of gap junctions between the somatic cells and oocyte because reducing junctional communication causes oocyte cAMP to decrease and meiosis to resume (Sela-Abramovich et al., 2006; Norris et al., 2008).

One possibility is that the signal that maintains meiotic arrest is cAMP that diffuses from the somatic cells to the oocyte (Anderson and Albertini, 1976) (see Discussion). Alternatively, because the primary cAMP phosphodiesterase in the oocyte is PDE3A (Masciarelli et al., 2004), which is inhibited by cGMP (Hambleton et al., 2005), cGMP from the somatic cells could enter the oocyte through the gap junctions, thus inhibiting PDE3A and maintaining meiotic arrest (Törnell et al., 1991). Consistent with this hypothesis, cGMP in the oocyte decreases as a function of time after isolation of the oocyte from the follicle, and injection of cGMP into the oocyte delays meiotic resumption (Törnell et al., 1990a). Inhibition of soluble guanylyl cyclase (Sela-Abramovich et al., 2008) or of inosine monophosphate dehydrogenase (needed for cGMP production) (Downs and Eppig, 1987; Eppig, 1991) causes meiotic resumption in follicle-enclosed oocytes, indicating that production of cGMP in the follicle is essential for the maintenance of meiotic arrest.

However, the hypothesis that cGMP from the somatic cells maintains meiotic arrest has not been directly tested; it is unknown whether gap junction closure lowers cGMP in the oocyte, or whether lowering oocyte cGMP lowers cAMP levels sufficiently to trigger meiotic resumption. It has also been proposed that instead of cGMP, phosphorylation could be the primary regulator of PDE3A in the oocyte (Han et al., 2006). Here, we directly test the possible role of cGMP in maintaining meiotic arrest.

Whether LH causes cAMP and cGMP in the oocyte to decrease to a level that allows meiotic resumption, and if so how this occurs, have also been uncertain. LH causes a rapid and almost complete closure of the gap junctions between the somatic cells in the follicle (Norris et al., 2008), such that if cGMP from the somatic cells enters

[1]Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06032, USA. [2]Cardiology Section, Veterans Affairs Salt Lake City Health Care System, Salt Lake City, UT 84148, and the Departments of Internal Medicine (Cardiology) and Pharmacology, University of Utah, Salt Lake City, UT 84132, USA. [3]Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA. [4]Institute of Pharmacology and Toxicology, University of Würzburg, D-97078 Würzburg, Germany.

*Authors for correspondence (e-mails: nikolaev@toxi.uni-wuerzburg.de; ljaffe@neuron.uchc.edu)

Accepted 1 April 2009

the oocyte prior to LH stimulation, the gap junction closure in response to LH should decrease cGMP, and indirectly cAMP, in the oocyte. In mouse and rat oocytes isolated from follicles after LH receptor stimulation, immunoassays have shown that cAMP has decreased (Schultz et al., 1983; Sela-Abramovich et al., 2006). However, the magnitude of these changes was difficult to interpret because the oocytes were removed from their follicles, which would decrease cAMP, and because phosphodiesterase inhibitors were present, which would increase cAMP. Thus, it is unknown whether the decreases in cAMP that were observed in response to LH receptor stimulation were sufficient to affect the activity of PKA, which requires cAMP in the range of 70-500 nM for half-maximal activity (Dostmann and Taylor, 1991; Viste et al., 2005). LH-stimulated decreases in cGMP occur in oocytes and whole follicles from hamsters (Hubbard, 1986), but whether the magnitude of these changes is physiologically significant and whether these decreases occur in other species are unknown. These are crucial issues because, as noted above, cGMP is not the only regulator of PDE3A; in addition, it is controversial whether relief of inhibitory signals from the somatic cells is the primary mode of LH action (see Eppig et al., 2004).

Because follicle integrity is essential for regulating oocyte cAMP and cGMP, understanding the function of cyclic nucleotides in the oocyte requires measurements within the intact follicle. Optical indicators based on Förster resonance energy transfer (FRET) (Nikolaev and Lohse, 2006; Nikolaev et al., 2006; Russwurm et al., 2007; Willoughby and Cooper, 2008) are ideal for this purpose. These sensors have been used in mouse oocytes (Webb et al., 2002; Webb et al., 2008), although not in oocytes within antral follicles. The sensor used in those studies, comprising fluorescent catalytic and regulatory subunits of PKA, has the disadvantage of inhibiting meiotic progression (Webb et al., 2002), probably because it elevates PKA activity (Goaillard et al., 2001). Other available sensors are not optimal for investigation of cAMP changes that control PKA-mediated events because their dynamic ranges do not correspond to that of PKA (Nikolaev and Lohse, 2006).

In the present study we developed a more sensitive FRET-based cAMP sensor and used it, as well as a cGMP sensor, to measure cAMP and cGMP in living follicle-enclosed mouse oocytes. We show that in response to LH, concentrations of both cAMP and cGMP decrease in the oocyte prior to nuclear envelope breakdown (NEBD) and within concentration ranges that are physiologically significant for regulation of PKA and PDE3A. The level of cGMP in the somatic cells of the follicle decreases in parallel. By injecting specific phosphodiesterases that hydrolyze cAMP and cGMP, and by applying inhibitors that control gap junction communication, we establish that cGMP from the somatic cells is essential for maintaining meiotic arrest and that LH causes meiosis to resume by reversing this inhibitory pathway.

MATERIALS AND METHODS

cAMP and cGMP sensors

To develop a FRET-based sensor with increased affinity for cAMP, we introduced a K405E point mutation into the EPAC2 (RAPGEF4) sequence of the previously described Epac2-camps sensor, which consists of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) linked by the cAMP-binding domain of EPAC2 (Nikolaev et al., 2004) (Fig. 1A). Similar mutation of the homologous residue Q270 in EPAC1 (RAPGEF3) increases the affinity for cAMP by ~2.5-fold (Dao et al., 2006). This new sensor, Epac2-camps300, was expressed in Sf9 cells and purified using Ni-NTA agarose (Qiagen, Valencia, CA, USA) and heparin sepharose; the protein was more than 90% pure.

In vitro measurements with the Epac2-camps and Epac2-camps300 sensors were performed as previously described (Nikolaev et al., 2004), with purified protein diluted in 5 mM Tris, 2 mM EDTA (pH 7.4) to a final concentration of 50 nM. The EC_{50} value for Epac2-camps in this buffer (~800-900 nM; Fig. 1B) (Nikolaev et al., 2004) was close to that previously determined in a buffer more closely resembling the cytosol (1100 nM) (Iancu et al., 2008). Emission spectra were measured using a fluorescence spectrometer (LS50B, Perkin Elmer Life Sciences, Waltham, MA, USA) with 436 nm excitation. YFP/CFP emission ratios were calculated by dividing fluorescence intensities measured at the 527 and 478 nm peaks, and were corrected for the spectral bleed-through of CFP into the YFP channel.

The cGi500 sensor for cGMP, which consists of YFP and CFP linked by the tandem cGMP-binding domains of cGMP-dependent protein kinase I, was constructed as previously described (Russwurm et al., 2007). Fluorescence spectra of the cGi500 sensor were measured using the supernatant from a homogenate of transiently transfected HEK293a cells in 5 mM Tris, 2 mM EDTA (pH 7.4) (see Fig. S1 in the supplementary material). The EC_{50} value in this buffer (~350 nM) was close to that reported in a higher ionic strength, chloride buffer (500 nM) (Russwurm et al., 2007). For microinjection, cGi500 RNA was post-transcriptionally polyadenylated (see Freudzon et al., 2005) and injected into oocytes at a final concentration of 40-100 μg/ml.

Culture and microinjection of antral follicle-enclosed mouse oocytes

Antral follicles with their associated theca cells (see Norris et al., 2008) were dissected from 22- to 24-day-old B6SJLF1 mice (Jackson Laboratory, Bar Harbor, ME, USA), as approved by the University of Connecticut Animal Care Committee. They were cultured for 24-32 hours on Millicell culture plates (PICMORG50, Millipore, Billerica, MA, USA) in media as previously described (Norris et al., 2008). Ovine LH (from A. F. Parlow, National Hormone and Peptide Program, Torrance, CA, USA) was used at 10 μg/ml. Quantitative microinjection of 4 or 10 pl (equivalent to 2 or 5% of the 200 pl volume of the oocyte) was carried out as previously described, with the follicles held between two coverslips spaced 200 μm apart (Jaffe and Terasaki, 2004; Jaffe et al., 2009). Follicles were kept in the injection slide for no more than 30 minutes; between injections, they were returned to Millicell culture plates. Proteins for injection were spin-dialyzed and concentrated in PBS.

Measurements of cAMP and cGMP in follicle-enclosed oocytes

FRET measurements were made 2.5-10 hours after injecting follicle-enclosed oocytes with Epac2-camps300 protein, or 19-26 hours after injecting cGi500 RNA. We used a Zeiss Pascal confocal system with a 20×/0.5 NA Neofluar objective, and a 440 nm laser (Toptica Photonics, Victor, NY, USA) for excitation. YFP and CFP emission filters were from Chroma Technology (Rockingham, VT, USA; HQ535/50M and HQ480/40M, with a 510DCLP dichroic). The microscope was focused on the oocyte equator, with the confocal pinhole set for a ~30 μm optical section. Images were collected at 2-10 second intervals. The laser attenuation was adjusted to avoid saturation. The follicles were held in an injection slide as described above, with humidified 5% CO_2 in air flowing through the chamber on the microscope stage at 30-34°C (see Jaffe et al., 2009).

YFP and CFP intensities were measured within a circular region of interest that was slightly smaller than the oocyte diameter. The percentage change in the YFP/CFP emission ratio following injection of cAMP or cGMP was calculated from values averaged over a 2-minute period before injection and over a 20-second period after injection. Measurements were corrected for minor autofluorescence and for spectral bleed-through of CFP into the YFP channel. Data analysis used the following software: MetaMorph (Molecular Devices, Downingtown, PA, USA), Excel (Microsoft, Redmond, WA, USA), Origin (OriginLab, Northampton, MA, USA) and InStat (GraphPad Software, San Diego, CA, USA). Data are reported as mean±s.e.m.; *n*=the number of oocytes tested. *P*-values were determined using an unpaired Student's *t*-test. The cAMP hydrolytic activity of PDE3A was calculated using the following equation for Michaelis-

Menten kinetics with cGMP as a competitive inhibitor (see Cook and Cleland, 2007) and values for K_m and K_i from Hambleton et al. (Hambleton et al., 2005): V/V_{max}=[cAMP]/{K_m(1+[cGMP]/K_i)+[cAMP]}.

PDE3A, PDE9A, CX37 antibody and other reagents

The catalytic domain (amino acids 665-1141) of human PDE3A was purified from bacteria. The protein was more than 90% pure and its activity, as assayed at 0.2 μM cAMP, was ~0.2 μmoles/minute/mg. Results similar to those described here were also obtained with a partially purified preparation of a form of PDE3A lacking the membrane-association region (PDE3A-94) (Hambleton et al., 2005). The catalytic domain (amino acids 181-506) of human PDE9A was purified from bacteria (Huai et al., 2004). The protein was more than 90% pure and had a V_{max} of 1.5 μmoles/minute/mg. A D293A point mutation in the PDE9A catalytic domain caused a 10,000-fold decrease in activity. An affinity-purified antibody against the C-terminal domain of CX37 was provided by Alex Simon (University of Arizona) (Simon et al., 2006). Cyclic nucleotides, ATP, carbenoxolone, milrinone and BSA were obtained from Sigma-Aldrich (St Louis, MO, USA), U0126 from EMD Chemicals (La Jolla, CA, USA) and non-immune IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

cGMP content of whole follicles

Antral follicles were dissected and cultured as described above. After washing in PBS, groups of ~20 follicles were solubilized in 100 μl 0.1 M HCl, sonicated, heated at 95°C for 2 minutes, then stored at –80°C before being analyzed using a cGMP immunoassay kit (Sigma-Aldrich, CG200); the cross-reactivity of this assay with GMP, GTP, cAMP and ATP is negligible (Sigma product information). The cellular volume per follicle was estimated to be ~20 nl, based on an average follicle diameter of ~360 μm; of the antral follicle volume, ~10% was estimated to be antral space.

RESULTS

Epac2-camps300, a new cAMP sensor that responds in the cAMP concentration range that regulates PKA

To determine whether LH reduces oocyte cAMP to a level that lowers PKA activity, we developed a new sensor, Epac2-camps300 (Fig. 1A), which shows 50% of its maximum change in the YFP/CFP emission ratio (EC_{50}) at ~320 nM cAMP (Fig. 1B), which is in the concentration range that half-maximally activates PKA enzymes. Epac2-camps300 was produced by making a K405E point mutation in the previously described Epac2-camps (Nikolaev et al., 2004), which has an EC_{50} of ~820 nM for cAMP (Fig. 1B). Epac2-camps300 was highly specific for cAMP, with an EC_{50} of ~14 μM for cGMP (Fig. 1C). ATP at 10 mM had no effect on Epac2-camps300 FRET. The cGMP concentration in mouse oocytes is ~900 nM (see below) and the ATP concentration is ~1 mM (Johnson et al., 2007). Thus, cytoplasmic cGMP and ATP should not interfere with the use of this sensor to measure changes in cAMP in the oocyte cytoplasm.

Measurement of cAMP changes in follicle-enclosed mouse oocytes

To establish a method for live cell measurements of changes in cAMP in antral follicle-enclosed oocytes, we microinjected the oocytes with Epac2-camps300 protein. Using a confocal microscope, we excited CFP and measured the ratio of YFP to CFP emission as an indicator of the concentration of cAMP in the oocyte (Fig. 2). To avoid variability due to the differing optical properties of individual follicles, we standardized each measurement by subsequently injecting 1 mM cAMP to saturate the sensor. This provided a measure of the percentage change in the YFP/CFP emission ratio between the baseline concentration of cAMP and that at the maximum of the concentration-response curve (Fig. 3A). The percentage change was the same using 2 or 5 μM sensor, indicating that the 5 μM Epac2-camps300 we used did not alter the basal concentration of free cAMP in the oocyte (see Fig. S2A in the supplementary material). Follicle-enclosed oocytes that were injected with Epac2-camps300 resumed meiosis in response to LH, and NEBD occurred with the same time course as in the absence of the sensor, beginning at ~2 hours after LH application (see Fig. S3A in the supplementary material).

LH causes a decrease in cAMP in the follicle-enclosed oocyte before NEBD

To investigate the effect of LH on oocyte cAMP, we measured the Epac2-camps300 YFP/CFP emission ratio from follicle-enclosed oocytes. After determining this baseline ratio, the follicles were injected with 1 mM cAMP to determine the percentage change in the ratio in going from baseline to the cAMP-saturated condition. For follicle-enclosed oocytes that had not been exposed to LH

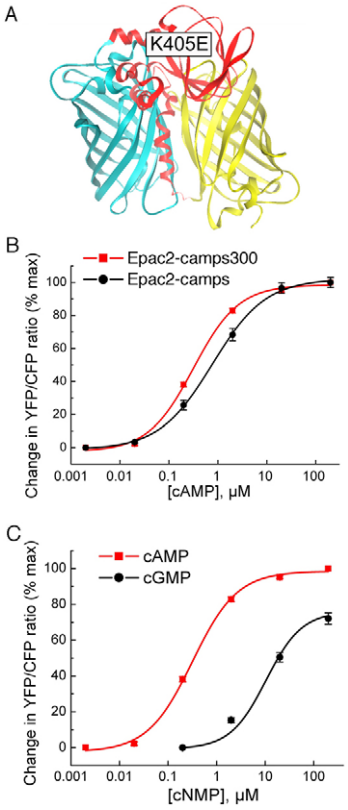

Fig. 1. In vitro characterization of the Epac2-camps300 sensor for cAMP. (**A**) Diagram of the sensor, showing cyan fluorescent protein (CFP, turquoise) and yellow fluorescent protein (YFP, yellow), linked by the EPAC2 (RAPGEF4) cAMP-binding domain (red) [constructed based on the crystal structures of EPAC2 (Protein Data Bank accession number 107F) and green fluorescent protein (Protein Data Bank accession number 1GFL)]. The location of the K405E point mutation introduced into the EPAC2 sequence of Epac2-camps to generate Epac2-camps300 is shown. Binding of cAMP decreases Förster resonance energy transfer (FRET) between CFP and YFP. (**B**) Concentration-response curves for Epac2-camps and Epac2-camps300 for cAMP. The EC_{50} (50% of the maximum change in the YFP/CFP emission ratio) values are 820±130 nM (mean±s.e.m., *n*=7) for Epac2-camps, and 320±18 nM (*n*=6) for Epac2-camps300. (**C**) Concentration-response curves for Epac2-camps300 for cAMP and cGMP. The EC_{50} value for cGMP is 14±4 μM (*n*=4).

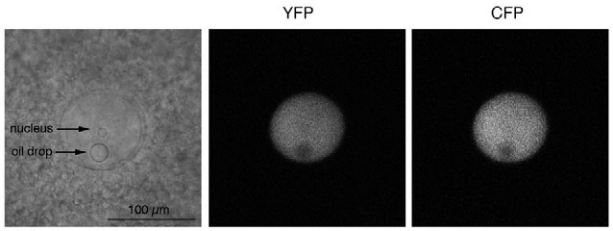

Fig. 2. Confocal images of an antral follicle-enclosed oocyte containing 5 μM Epac2-camps300. The oil drop is present as a result of microinjection. Upon excitation of CFP, the ratio of YFP to CFP emission was measured as an indicator of the concentration of cAMP in the oocyte.

(basal), the YFP/CFP emission ratio changed by 15% (Fig. 3A,D). By contrast, for follicles that had been exposed to LH for 1-1.4 hours, the ratio changed by 26% (Fig. 3B,D). For follicles exposed to LH for 5 hours, the ratio changed by 33% (Fig. 3C,D). These measurements showed that the concentration of cAMP in the follicle-enclosed oocyte decreases in response to LH and that this occurs before NEBD.

The cAMP decrease in response to LH occurs in a concentration range that would decrease PKA activity

To evaluate whether the cAMP decrease in response to LH occurs in the regulatory range for PKA, we determined the absolute concentrations of cAMP in follicle-enclosed oocytes before and after LH exposure. For this purpose, we needed to know the percentage change in the YFP/CFP emission ratio in the oocyte over the dynamic range of the Epac2-camps300 sensor. We first injected the catalytic domain of the high-affinity cAMP phosphodiesterase PDE3A (K_m=~90 nM) (Hambleton et al., 2005) to lower cAMP to a minimum level. One hour later, we injected 1 mM cAMP to saturate the sensor and measured the percentage change in the YFP/CFP emission ratio. Using either 100 or 200 μg/ml PDE3A, the subsequent injection of 1 mM cAMP caused the same change in the YFP/CFP emission ratio (~36%, Fig. 4A,B), indicating that the amount of PDE3A used was sufficient to obtain a maximum response, and that the change in the YFP/CFP emission ratio over the dynamic range of Epac2-camps300 in the oocyte is ~36%. We then used this value, together with the concentration-response curve obtained in vitro (Fig. 1B), to determine the concentrations of cAMP in the oocyte before and after LH exposure (Fig. 4C).

Based on this analysis, the basal concentration of cAMP in antral follicle-enclosed oocytes is ~660 nM (Fig. 4C). At 1-1.4 hours after LH, the cAMP concentration is ~140 nM, and at 5 hours it is ~40 nM (Fig. 4C). The cAMP decrease from ~660 to ~140 nM would decrease the activity of PKA in the oocyte. Mouse oocytes contain both PKAI and PKAII, although the relative amounts of these two forms have not been determined (Newhall et al., 2006). PKAII is half-maximally activated at ~500 nM cAMP (Dostmann and Taylor, 1991; Viste et al., 2005), and based on data in these publications a decrease in cAMP from ~660 to ~140 nM would reduce the activity of PKAII by 90%. PKAI activity would also decrease, although the extent of this decrease is unknown, as values for the cAMP concentrations that half-maximally activate PKAI vary from 70 to 500 nM depending on the type and concentration of the substrate (Viste et al., 2005).

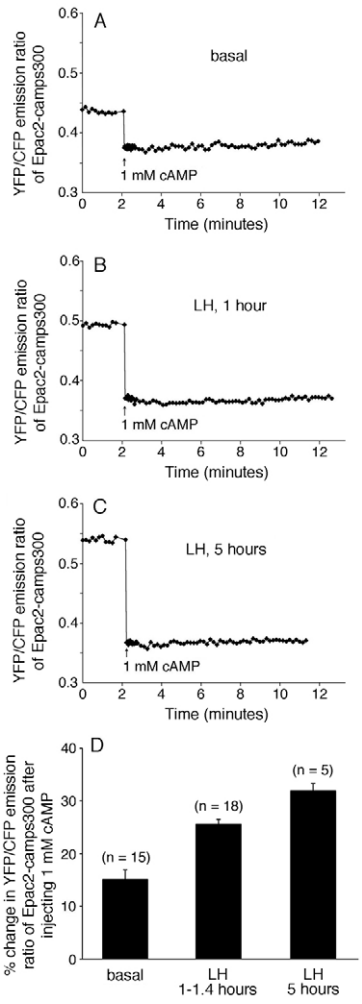

Fig. 3. The concentration of cAMP in follicle-enclosed oocytes decreases in response to luteinizing hormone. (**A-C**) The YFP/CFP emission ratio from follicle-enclosed mouse oocytes containing Epac2-camps300, before and after injecting 1 mM cAMP, for no treatment (basal) (A) and for ~1 hour (B) or ~5 hours (C) after applying luteinizing hormone (LH). (**D**) Percentage change in the YFP/CFP emission ratio in response to injection of 1 mM cAMP for follicle-enclosed oocytes with or without LH exposure. The percentage change was 15.1±1.8 (*n*=15) for no LH, 25.6±0.9 (*n*=18) for 1-1.4 hours LH, and 31.9±1.4 (*n*=5) for 5 hours LH.

LH causes a decrease in cGMP in the follicle-enclosed oocyte before NEBD and within a concentration range that would increase PDE3A activity

To investigate whether the concentration of cGMP in the follicle-enclosed mouse oocyte decreases in response to LH, we used a FRET-based cGMP sensor, cGi500 (Russwurm et al., 2007). cGi500 showed an EC_{50} of ~350 nM for cGMP and little or no response to cAMP up to 20 μM (see Fig. S1 in the supplementary material) (Russwurm et al., 2007). In follicle-enclosed oocytes that were injected with cGi500 RNA and then cultured overnight, LH caused meiotic resumption with the same time course as in the absence of the sensor (see Fig. S3B in the supplementary material). The CFP fluorescence of oocytes expressing this protein was close to that of oocytes containing 5 μM Epac2-camps300, indicating that the concentration of cGi500 was also in the range of 5 μM.

For cGMP measurements, the YFP/CFP emission ratio was determined for follicle-enclosed oocytes expressing cGi500. The oocytes were injected with 1 mM cGMP to determine the percentage change in the ratio. For follicles that had not been exposed to LH (basal), the YFP/CFP emission ratio changed by 14% (Fig. 5A,C). By contrast, for follicles that had been exposed to LH for 1-1.3 or 5 hours, the ratio changed by 30% (Fig. 5B,C). These measurements showed that LH causes a decrease in cGMP in follicle-enclosed oocytes and that the decrease precedes NEBD.

To calibrate the concentration of cGMP in the follicle-enclosed oocyte, we used a similar procedure as for cAMP. To determine the percentage change in the YFP/CFP emission ratio for cGi500 over its dynamic range in vivo, we injected follicle-enclosed oocytes with the catalytic domain of the high-affinity cGMP-specific phosphodiesterase PDE9A (K_m=140 nM) (Huai et al., 2004), and 1 hour later injected 1 mM cGMP (Fig. 6A). A similar percentage change in the YFP/CFP emission ratio (~35%) was obtained with injections of 180 or 360 μg/ml PDE9A, indicating that sufficient PDE9A had been injected to obtain a maximum response (Fig. 6B). Based on the calibration curve shown in Fig. 6C, the basal concentration of free cGMP in the oocyte is ~900 nM. At 1-1.3 hours and at 5 hours after LH application, the cGMP concentration is ~40 nM. Comparison of measurements from individual oocytes that expressed somewhat different amounts of the cGi500 sensor showed that the sensor did not alter the concentration of cGMP in the oocyte (see Fig. S2B,C in the supplementary material).

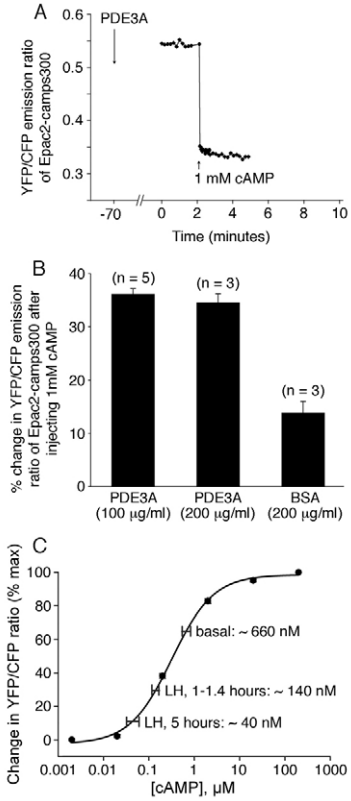

Fig. 4. Calibration of the cAMP concentrations in follicle-enclosed oocytes with or without LH treatment. (**A**,**B**) Determination of the percentage change in the YFP/CFP emission ratio for Epac2-camps300 in going from a minimum to a maximum cAMP concentration in vivo. (A) Record from a follicle-enclosed mouse oocyte that was injected with 5 μM Epac2-camps300 plus 100 μg/ml PDE3A catalytic domain (to lower cAMP to a minimum level), and then ~1 hour later injected with 1 mM cAMP. (B) Percentage change in YFP/CFP emission ratio in follicle-enclosed oocytes injected with PDE3A, or BSA control, and then ~1 hour later injected with 1 mM cAMP. Injection of 100-200 μg/ml PDE3A lowered the YFP/CFP emission ratio by 35.5±1.0% (n=8). (**C**) cAMP concentrations in follicle-enclosed oocytes with or without LH treatment, as calculated using the in vitro concentration-response curve for Epac2-camps300 (replotted here from Fig. 1B), the 35.5% value for the maximum change in YFP/CFP emission ratio for Epac2-camps300 in vivo (A,B), and the data presented in Fig. 3. The following example illustrates how these calculations were made. For the measurement shown in Fig. 3A, the change in YFP/CFP emission ratio in going from the baseline level to that after injecting 1 mM cAMP was 14%. Using the percentage change in YFP/CFP ratio over the dynamic range of the sensor in vivo, shown above to be 35.5%, we calculated the percentage change in YFP/CFP ratio corresponding to a change from ~0 mM cAMP to the baseline cAMP level in Fig. 3A: 35.5–14=21.5%. 21.5/35.5=61% of the maximum change in YFP/CFP ratio over the dynamic range of the sensor. 61% on the y-axis of Fig. 4C corresponds to 540 mM cAMP on the x-axis, as determined using Origin software. The horizontal bars in C represent the mean±s.e.m. for the cAMP concentrations calculated for the sets of measurements summarized in Fig. 3D. Basal, 660±110 nM (n=15); 1-1.4 hours, 140±18 nM (n=18); 5 hours, 43±17 nM (n=5). The cAMP concentrations at 1-1.4 and 5 hours after LH are significantly different from the basal value (P<0.0001 and P=0.005, respectively), and from each other (P<0.01).

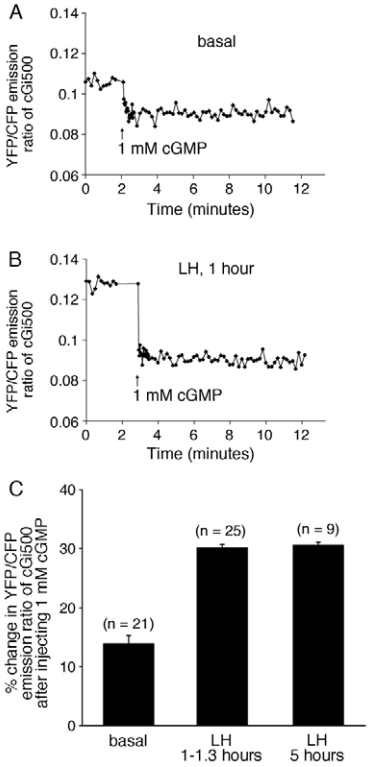

Fig. 5. The concentration of cGMP in follicle-enclosed oocytes decreases in response to LH. (**A**,**B**) The YFP/CFP emission ratio from follicle-enclosed mouse oocytes containing cGi500, before and after injecting 1 mM cGMP, for no treatment (basal) (A) and for ~1 hour after applying LH (B). (**C**) Percentage change in the YFP/CFP emission ratio for cGi500 in response to injection of 1 mM cGMP, for follicle-enclosed oocytes with or without LH treatment. The percentage change in YFP/CFP emission ratio was 13.9±1.4 (n=21) for no LH, 30.1±0.6 (n=25) for 1-1.3 hours LH, and 30.6±0.5 (n=9) for 5 hours LH.

The basal cGMP concentration in the oocyte of ~900 nM would competitively inhibit the hydrolysis of cAMP by PDE3A, whereas at the ~40 nM level after LH there would be less inhibition (Hambleton et al., 2005). Based on the enzymatic properties of PDE3A (K_m for cAMP=90 nM; K_i for cGMP=20 nM) (Hambleton et al., 2005), and at the initial cAMP concentration (~660 nM), the drop in cGMP would increase cAMP-hydrolytic activity from 14 to 71% of the maximum (see Materials and methods). This 5-fold increase in PDE3A activity would decrease oocyte cAMP, leading to meiotic resumption.

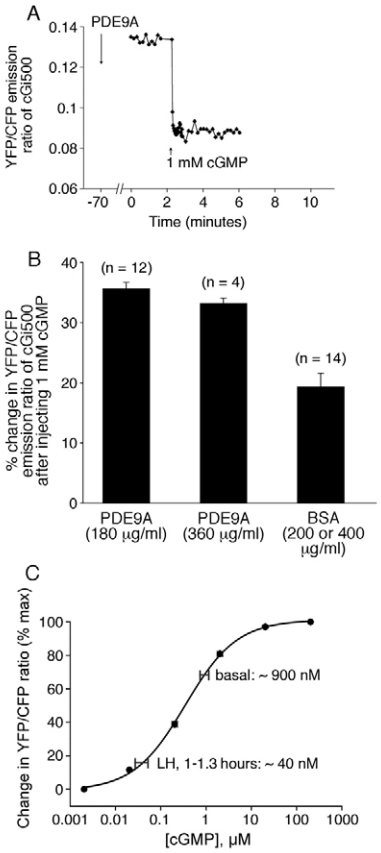

Fig. 6. Calibration of cGMP concentrations in follicle-enclosed oocytes with or without LH treatment. (**A**,**B**) Determination of the percentage change in the YFP/CFP emission ratio for cGi500 in going from a minimum to a maximum cGMP concentration in vivo. (A) Record from a follicle-enclosed mouse oocyte containing cGi500 that was injected with 360 μg/ml PDE9A catalytic domain (to lower cGMP to a minimum level) and ~1 hour later injected with 1 mM cGMP. (B) Percentage change in YFP/CFP emission ratio in follicle-enclosed oocytes injected with PDE9A, or BSA control, and then ~1 hour later injected with 1 mM cGMP. Injection of 180-360 μg/ml PDE9A lowered the YFP/CFP emission ratio by 35.0±0.9% (n=16). (**C**) cGMP concentrations in follicle-enclosed oocytes with or without LH treatment, as calculated by the procedure described in the legend to Fig. 4C, using the in vitro concentration-response curve for cGi500 (replotted here from Fig. S1 in the supplementary material), the 35.0% value for the maximum change in YFP/CFP emission ratio for cGi500 in vivo (A,B), and the data presented in Fig. 5. The horizontal bars in C represent the mean±s.e.m. for the cGMP concentrations calculated for the set of measurements summarized in Fig. 5C. Basal, 890±150 nM (n=21); 1-1.3 hours, 39±7 nM (n=25); 5 hours, 30±5 nM (n=9); for clarity, only the basal and 1-1.3 hour values are shown on the graph. The cGMP concentrations at 1-1.3 and 5 hours after LH are significantly different from the basal value (P<0.0001 and P=0.001, respectively), but not from each other (P=0.5).

Decreasing cGMP in follicle-enclosed oocytes causes cAMP levels to decrease

To test directly whether decreasing cGMP would decrease cAMP in the oocyte, we injected follicle-enclosed oocytes with a mixture of the catalytic domain of PDE9A and the cAMP-specific sensor Epac2-camps300. When measured ~1 hour later, cAMP in the oocyte had decreased to ~50 nM (Fig. 7A,B). The effect was not due to hydrolysis of cAMP by PDE9A because its K_m for cAMP is 180 μM, which is more than 1000-fold higher than that for cGMP (140 nM), and its V_{max} for cAMP is 20-fold lower than for cGMP (Huai et al., 2004). These findings indicate that a high concentration of cGMP is required to maintain the high concentration of cAMP in the prophase-arrested oocyte, and that the decrease in cGMP in response to LH is sufficient to decrease cAMP.

Decreasing cGMP in follicle-enclosed oocytes causes PDE3-dependent meiotic resumption

The PDE9A catalytic domain caused NEBD within ~2 hours after its injection into antral follicle-enclosed oocytes and the response was concentration dependent (Fig. 8A). By contrast, a control injection of a point-mutated form of PDE9A that lacks catalytic activity (D293A) did not cause NEBD. NEBD was also stimulated by injection of the catalytic domain of PDE3A, which hydrolyzes cAMP and, at a lesser rate, cGMP (Lugnier, 2006) (Fig. 8B).

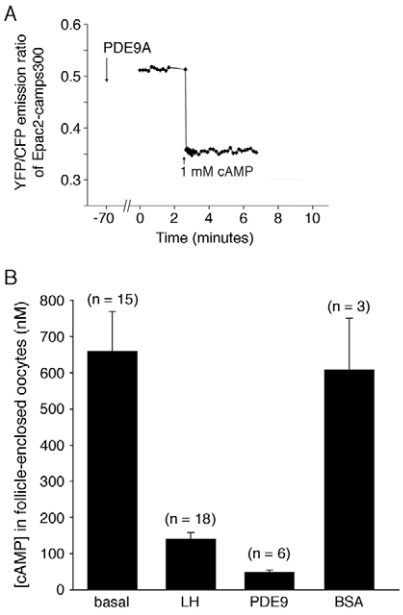

Fig. 7. Decreasing cGMP by injecting follicle-enclosed oocytes with the catalytic domain of PDE9A decreases cAMP. (**A**) Determination of the percentage change in the YFP/CFP emission ratio for Epac2-camps300 in a follicle-enclosed mouse oocyte that was injected with 180 μg/ml PDE9A catalytic domain and then ~1 hour later injected with 1 mM cAMP. The cAMP injection lowered the YFP/CFP emission ratio by 31.2±0.7% (n=6). (**B**) cAMP concentrations after injection of PDE9A, or BSA control (200 μg/ml), determined using the calibration curve in Fig. 4C. cAMP concentrations for basal and 1-1.4 hour LH conditions, from Fig. 4C, are shown for comparison.

PDE9A was only effective in causing meiotic resumption if PDE3A in the oocyte was active; in the presence of the PDE3 inhibitor milrinone (Lugnier, 2006), which does not inhibit PDE9A (Wunder et al., 2005), meiotic resumption in response to PDE9A injection was reversibly inhibited (Fig. 8C). This result indicates that the resumption of meiosis that occurs in response to a decrease in cGMP results from alleviation of PDE3A inhibition by cGMP, rather than through the interaction of cGMP with other cGMP-binding proteins such as cGMP-dependent protein kinases.

Inhibition of gap junction permeability in the follicle decreases cGMP in the oocyte

To investigate the effect of gap junction closure on cGMP in follicle-enclosed oocytes, we applied the gap junction inhibitor carbenoxolone (CBX) at 100 μM to follicles containing oocytes expressing the cGi500 sensor. Under these conditions, CBX closes gap junctions throughout the follicle by 1 hour and NEBD occurs at 1-2.5 hours (see Norris et al., 2008). At 1-1.5 hours after applying CBX, cGMP in the oocyte had decreased to ~90 nM (Fig. 9A,C), which would cause a ~4-fold increase in the cAMP-hydrolytic activity of PDE3A.

Gap junction communication between the oocyte and somatic cells can also be decreased by injection of follicle-enclosed oocytes with an antibody that specifically recognizes connexin 37 (CX37; GJA4 – Mouse Genome Informatics) (Norris et al., 2008), which is the predominant connexin in the gap junctions between the oocyte and cumulus cells (Simon et al., 1997). Like CBX, the CX37 antibody causes meiotic resumption (Norris et al., 2008). NEBD occurs between 6 and 12 hours after injection; the lag time indicates that the antibody most likely affects CX37 turnover, rather than

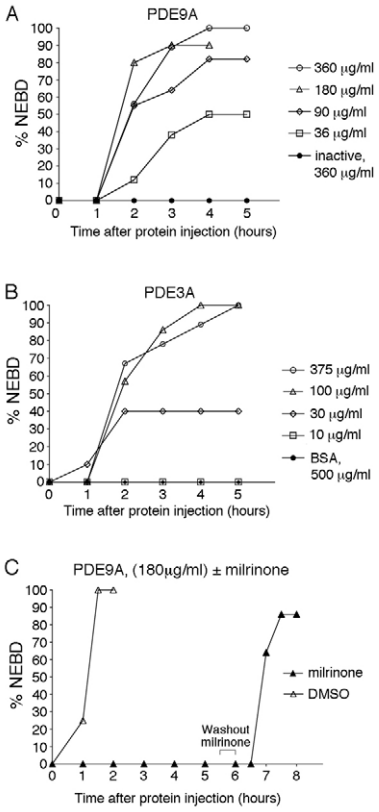

Fig. 8. Decreasing cGMP by injecting follicle-enclosed oocytes with the catalytic domain of PDE9A causes PDE3-dependent meiotic resumption. (**A**,**B**) Time course of nuclear envelope breakdown (NEBD) in response to injection of various concentrations of (A) the catalytic domain of PDE9A or a catalytically inactive mutant (D293A), or (B) the catalytic domain of PDE3A or BSA control. (**C**) Meiotic resumption in response to injection of PDE9A is reversibly inhibited by milrinone, an inhibitor of PDE3. Follicles were preincubated for 1-2 hours with 100 μM milrinone or with a control solution containing 0.5% DMSO; then, the follicle-enclosed mouse oocytes were injected with 180 μg/ml PDE9A and observed for NEBD. Five hours later, the follicles were washed into milrinone-free medium. Each curve represents the results from injection of 7-14 follicle-enclosed oocytes.

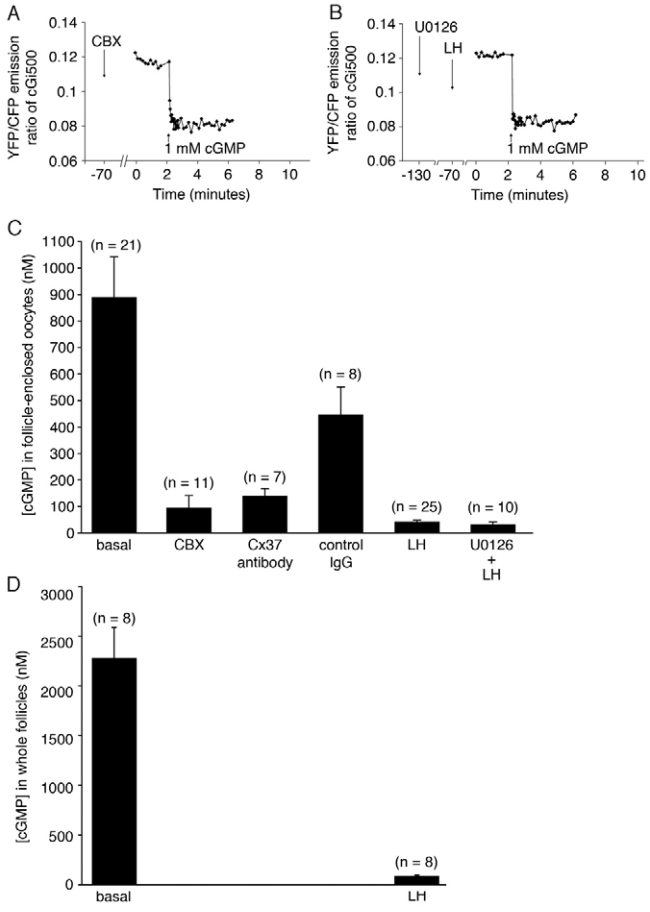

Fig. 9. cGMP concentrations in follicle-enclosed oocytes treated with reagents that affect gap junction closure, and in follicle-enclosed oocytes and whole follicles in response to LH. (**A**,**B**) The YFP/CFP emission ratio from follicle-enclosed mouse oocytes containing the cGMP sensor cGi500, before and after injecting 1 mM cGMP, for follicles treated with 100 μM CBX (A), or LH plus 10 μM U0126 (B). (**C**) cGMP concentrations in follicle-enclosed oocytes after various treatments (see text), as determined from measurements as in A,B and the calibration curve in Fig. 6C. The values for basal and 1-1.3 hour LH conditions are from Fig. 6C. Oocytes had not undergone NEBD at the time of the measurements. (**D**) cGMP concentrations in whole follicles with or without treatment for 1 hour with LH (mean±s.e.m. for *n* independent assays). Basal, 2300±310 nM (*n*=8); 1 hour LH, 85±14 nM (*n*=8).

directly inhibiting channel permeability (Norris et al., 2008). In accordance with the stimulation of meiotic resumption, the CX37 antibody (2 μM) caused a decrease in cGMP in the oocyte when measured 5-6 hours after injection (Fig. 9C).

These results support the conclusion that diffusion from the somatic cells to the oocyte of cGMP, or of a small molecule required for cGMP accumulation, is required to maintain prophase arrest. LH-induced closure of gap junctions inhibits this diffusion and is a stimulus for meiotic resumption in the oocyte.

LH also causes cGMP to decrease in the oocyte by a second pathway that is independent of gap junction closure

LH signaling leads to meiotic resumption by two parallel pathways: mitogen-activated protein kinase (MAPK)-dependent closure of gap junctions via phosphorylation of connexin 43 (CX43; GJA1 – Mouse Genome Informatics), which is the predominant connexin in the gap junctions between the somatic cells, and another pathway that is independent of both MAPK activation and the resulting gap junction closure (Norris et al., 2008). The existence of this second pathway was demonstrated by the finding that inhibition of MAPK activation by 10 μM U0126 prevents LH-stimulated gap junction closure but not meiotic resumption (Norris et al., 2008) (see Fig. S4 in the supplementary material).

To investigate whether LH can decrease oocyte cGMP levels without closing gap junctions, we applied LH to follicles in the presence of 10 μM U0126, and 1-1.5 hours later measured cGMP in the follicle-enclosed oocytes. The cGMP concentration under these conditions was ~30 nM, the same as that in follicle-enclosed oocytes exposed to LH without U0126 (Fig. 9B,C). Thus, both of the pathways by which LH causes meiotic resumption cause a decrease in cGMP prior to NEBD.

LH decreases cGMP in the somatic cells of the follicle

We next investigated whether the cGMP concentration in the somatic cells of an unstimulated follicle is similar to that in the oocyte, and whether LH causes a decrease in somatic cell cGMP that could account for the gap junction closure-independent decrease in oocyte cGMP. A decrease in cGMP concentration, to 40% of the initial value, was previously reported in hamster follicles exposed to LH (Hubbard, 1986).

In a mechanically isolated follicle, ~90% of the cellular volume comprises the follicle itself, with the remainder comprising adhering theca and interstitial cells that are not connected by gap junctions to the follicle and are not considered to be part of the follicle. These adhering cells cannot be removed without disrupting the follicle. Within the follicle, ~99% of the ~20 nl cellular volume is due to the somatic cells, compared with ~0.2 nl for the oocyte. Therefore, determination of cGMP in isolated follicles provides an approximate measurement of cGMP in the somatic cells of the follicle.

Basal cGMP in isolated follicles was determined by immunoassay to be ~2 μM, and 1 hour after applying LH it had decreased to ~80 nM (Fig. 9D). Considering the different methods of measurement that were used, and the contribution of extrafollicular cells to the whole-follicle measurements, the values that we obtained for cGMP in whole follicles and in follicle-enclosed oocytes are similar (compare Fig. 9C with 9D). These results support the conclusion that the decrease in cGMP in the somatic cells of the follicle contributes, via gap junctions, to the decrease in cGMP in the oocyte.

DISCUSSION

Monitoring cAMP and cGMP levels in intact follicle-enclosed oocytes

Ovarian follicles are multicellular complexes that consist of oocytes surrounded by somatic cells; all of these cells are interconnected by dynamically regulated gap junctions and function together to regulate meiosis in the oocyte. Here we have developed FRET-based methods to measure concentrations of cytosolic cAMP and cGMP in follicle-enclosed oocytes, allowing us to evaluate how cyclic nucleotide-regulated enzymes function in a physiologically intact tissue to control cell cycle progression.

cGMP from the somatic cells maintains meiotic arrest in the oocyte by inhibiting PDE3A

Results described here demonstrate that elevated cGMP in the oocyte is required to maintain prophase arrest, and that the suppression of meiotic progression by cGMP is due to its inhibitory effect on PDE3A hydrolysis of cAMP, thus keeping PKA active. The somatic cells maintain the arrest by providing the oocyte with cGMP through gap junctions (see Fig. 10). If guanylyl cyclases are present at relatively high levels in the somatic cells, but at relatively low levels in the oocyte, the oocyte would be dependent on the somatic cells for its supply of cGMP; guanylyl cyclase agonists have inhibitory effects on spontaneous meiotic resumption in cumulus-oocyte complexes, but not in isolated oocytes, suggesting that this is likely (Törnell et al., 1990b; Bu et al., 2004).

Cyclic AMP diffusion into the oocyte from the somatic cells could also contribute to maintaining meiotic arrest (Anderson and Albertini, 1976). However, this idea is difficult to reconcile with evidence showing that when cAMP generation in the oocyte is inhibited, by preventing the function of GPR3 or G_s in the oocyte, cAMP from the somatic cells is insufficient to maintain cAMP in the oocyte at a level that prevents meiotic resumption (Mehlmann et al., 2002; Mehlmann, 2005b). The concentration of cAMP in the somatic cells of an unstimulated follicle probably varies in different microdomains; it appears to be low in regions that regulate PKA (Hunzicker-Dunn, 1981; Panigone et al., 2008), but higher elsewhere (Schultz et al., 1983; Hashimoto et al., 1985; Hsieh et al., 2007). This issue requires further study. If a somatic cell-oocyte cAMP gradient is present, selective permeability of CX37 for cGMP over cAMP is a possible mechanism that could limit diffusion of cAMP through the CX37 junctions at the surface of the oocyte. Although the cGMP/cAMP permeability of CX37 has not been investigated, some other gap junctions are known to be selectively permeable to cGMP (Bevans et al., 1998; Locke et al., 2004).

LH lowers oocyte cGMP and stimulates meiotic resumption by decreasing somatic cell cGMP and gap junction permeability

Receptors for LH are present in the mural granulosa and theca cells, but not in the cumulus cells or oocyte (Amsterdam et al., 1975). LH receptor activation stimulates G_s and activates adenylyl cyclase (Hunzicker-Dunn and Mayo, 2006), and as a consequence elevates cAMP levels (Schultz et al., 1983; Hashimoto et al., 1985; Hsieh et al., 2007) and PKA activity (Hunzicker-Dunn, 1981; Panigone et al., 2008) in the mural granulosa cells. One way that the LH signal is conveyed into the follicle interior is by the PKA-dependent release of peptides that activate EGF receptors, which in turn activate MAPK (Panigone et al., 2008). MAPK phosphorylates and thus closes the CX43 gap junctions throughout the somatic compartment, although the permeability of the CX37 gap junctions between the cumulus cells and oocyte remains unchanged (Sela-Abramovich et

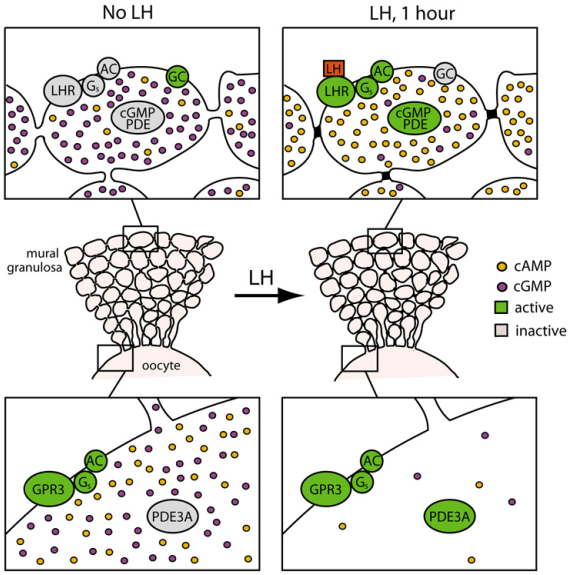

Fig. 10. Working hypothesis for how meiotic arrest is maintained and how LH causes meiotic resumption in follicle-enclosed mouse oocytes. Diagramatic representations of low-magnification views of a section of mouse follicle (center), and higher-magnification views of a mural granulosa cell (top) and the oocyte (bottom). (Left) No LH. The cAMP concentration in the mural granulosa cells is relatively low because the LH receptor (LHR)/G_s/adenylyl cyclase (AC) system is inactive. The cGMP concentration is high, owing to active guanylyl cyclase (GC) and inactive cGMP phosphodiesterase (cGMP PDE). In the oocyte, the concentration of cGMP is high owing to diffusion of cGMP from the somatic cells through the gap junctions. The concentration of cAMP is high owing to the constitutive activity of GPR3 and the inhibition of PDE3A by cGMP. Elevated cAMP maintains meiotic arrest. (Right) One hour after LH application. Activation of the LH receptor activates G_s and adenylyl cyclase, elevating cAMP levels in the mural granulosa cells. This initiates a signaling pathway that closes gap junctions throughout the somatic compartment (see text). LH also causes cGMP levels in the somatic cells to decrease by decreasing guanylyl cyclase activity and/or by increasing cGMP phosphodiesterase activity. As a result of the closure of gap junctions and the decrease in cGMP in the somatic cells, cGMP levels in the oocyte decrease, inhibition of PDE3A is relieved, cAMP decreases and meiosis resumes.

al., 2005; Sela-Abramovich et al., 2006; Norris et al., 2008). Our results indicate that gap junction closure reduces the flux of cGMP from the somatic cells to the oocyte (Fig. 10).

In addition, LH decreases somatic cell cGMP, such that less cGMP would move from the somatic cells into the oocyte, even if gap junctions remain open (Fig. 10). The decrease in cGMP in the somatic cells could result from inhibition of a guanylyl cyclase or from stimulation of a cGMP phosphodiesterase. There is some evidence for regulation of the cyclase rather than the phosphodiesterase (Patwardhan and Lanthier, 1984), although it remains to be determined which particular guanylyl cyclases and/or phosphodiesterases are involved, how they are regulated, and whether interfering with LH-induced changes in their activity prevents meiotic resumption. It is also unknown whether EGF receptor activation functions in the pathway by which LH causes cGMP levels to decrease in the somatic cells.

Mechanisms other than the decrease in cGMP might also contribute to the decrease in oocyte cAMP in response to LH. Evidence argues against regulation of cAMP production by inhibition of GPR3/G_s signaling or stimulation of a G_i- or Ca^{2+}-mediated pathway in the oocyte (Mehlmann et al., 2006; Norris et al., 2007), but cGMP-independent stimulation of PDE3A (Richard et al., 2001; Han et al., 2006) or additional LH signaling pathways (Eppig and Downs, 1987; Kalous et al., 2006; Chen and Downs, 2008; Kawamura et al., 2009) might function in parallel with cGMP regulation. However, our results indicate that the decrease in cGMP in the oocyte is sufficient to account for the stimulation of meiotic resumption by LH.

We thank Leon Freudzon and Nikolai Artemyev for participation in preliminary experiments; Alex Simon for the CX37 antibody; A. F. Parlow for LH; Marco Conti, Ann Cowan, John Eppig, Alexei Evsikov, Paul Lampe, Martin Lohse, Gail Mandel, Melina Schuh and Mark Terasaki for helpful discussions and comments on the manuscript; Paul Lampe (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) for the immunoblotting and densitometry in connection with the results shown in Fig. S4 in the supplementary material; and Martina Fischer, Christian Dees and Amber Selko for technical assistance. This work was supported by grants from the NIH to L.A.J. (HD014939, DK073499) and H.K. (GM59791), and from the Interdisziplinares Zentrum fuer klinische Forschung Wuerzburg (IZKF E-40) and the Deutsche Forschungsgemeinschaft (SFB487) to V.O.N., and by medical research funds from the United States Department of Veterans Affairs and grants from the American Heart Association and the Leducq Foundation (06 CVD 02) to M.A.M. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1869/DC1

References

Amsterdam, A., Koch, Y., Lieberman, M. E. and Lindner, H. R. (1975). Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. *J. Cell Biol.* **67**, 894-900.

Anderson, E. and Albertini, D. F. (1976). Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J. Cell Biol.* **71**, 680-686.

Bevans, C. G., Kordel, M., Rhee, S. K. and Harris, A. L. (1998). Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J. Biol. Chem.* **273**, 2808-2816.

Bu, S., Xie, H., Tao, Y., Wang, J. and Xia, G. (2004). Nitric oxide influences the maturation of cumulus cell-enclosed mouse oocytes cultured in spontaneous maturation medium and hypoxanthine-supplemented medium through different signaling pathways. *Mol. Cell. Endocrinol.* **223**, 85-93.

Chen, J. and Downs, S. M. (2008). AMP-activated protein kinase is involved in hormone-induced mouse oocyte meiotic maturation in vitro. *Dev. Biol.* **313**, 47-57.

Cho, W. K., Stern, S. and Biggers, J. D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. *J. Exp. Zool.* **187**, 383-386.

Cook, P. F. and Cleland, W. W. (2007). In *Enzyme Kinetics and Mechanism*, p. 404. New York: Garland Science Publishing.

Dao, K. K., Teigen, K., Kopperud, R., Hodneland, E., Schwede, F., Christensen, A. E., Martinez, A. and Doskeland, S. O. (2006). Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition. *J. Biol. Chem.* **281**, 21500-21511.

Dostmann, W. R. G. and Taylor, S. S. (1991). Identifying the molecular switches that determine whether (R_p)-cAMPS functions as an antagonist or an agonist in the activation of cAMP-dependent protein kinase I. *Biochemistry* **30**, 8710-8716.

Downs, S. M. and Eppig, J. J. (1987). Induction of mouse oocyte maturation in vivo by perturbants of purine metabolism. *Biol. Reprod.* **36**, 431-437.

Eppig, J. J. (1991). Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte-granulosa cell complexes developed in vitro from preantral follicles. *Biol. Reprod.* **45**, 824-830.

Eppig, J. J. and Downs, S. M. (1987). The effect of hypoxanthine on mouse oocyte growth and development in vitro: maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. *Dev. Biol.* **119**, 313-321.

Eppig, J. J., Viveiros, M. M., Marin-Bivens, C. and De La Fuente, R. (2004). Regulation of mammalian oocyte maturation. In *The Ovary* (ed. P. C. K. Leung and E. Y. Adashi), 2nd edn, pp. 113-129. San Diego, CA: Elsevier/Academic Press.

Freudzon, L., Norris, R. P., Hand, A. R., Tanaka, S., Saeki, Y., Jones, T. L. Z., Rasenick, M. M., Berlot, C. H., Mehlmann, L. M. and Jaffe, L. A. (2005). Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the G_s G protein. *J. Cell Biol.* **171**, 255-265.

Goaillard, J.-M., Vincent, P. and Fischmeister, R. (2001). Simultaneous measurements of intracellular cAMP and L-type Ca^{2+} current in single frog ventricular myocytes. *J. Physiol.* **530**, 79-91.
Hambleton, R., Krall, J., Tikishvili, E., Honeggar, M., Ahmad, F., Manganiello, V. C. and Movsesian, M. A. (2005). Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium. *J. Biol. Chem.* **280**, 39168-39174.
Han, S. J., Vaccari, S., Nedachi, T., Andersen, C. A., Kovacina, K. S., Roth, R. A. and Conti, M. (2006). Protein kinase B/Akt phosphorylation of PDE3A and its role in mammalian oocyte maturation. *EMBO J.* **25**, 5716-5725.
Hashimoto, N., Kishimoto, T. and Nagahama, Y. (1985). Induction and inhibition of meiotic maturation in follicle-enclosed mouse oocytes by forskolin. *Dev. Growth Differ.* **27**, 709-716.
Hinckley, M., Vaccari, S., Horner, K., Chen, R. and Conti, M. (2005). The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. *Dev. Biol.* **287**, 249-261.
Horner, K., Livera, G., Hinckley, M., Trinh, K., Storm, D. and Conti, M. (2003). Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Dev. Biol.* **258**, 385-396.
Hsieh, M., Lee, D., Panigone, S., Horner, K., Chen, R., Theologis, A., Lee, D. C., Threadgill, D. W. and Conti, M. (2007). Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. *Mol. Cell. Biol.* **27**, 1914-1924.
Huai, Q., Wang, H., Zhang, W., Colman, R. W., Robinson, H. and Ke, H. (2004). Crystal structure of phosphodiesterase 9 shows orientation variation of inhibitor 3-isobutyl-1-methylxanthine binding. *Proc. Natl. Acad. Sci. USA* **101**, 9624-9629.
Hubbard, C. J. (1986). Cyclic AMP changes in the component cells of Graafian follicles: possible influences on maturation in the follicle-enclosed oocytes of hamsters. *Dev. Biol.* **118**, 343-351.
Hunzicker-Dunn, M. (1981). Selective activation of rabbit ovarian protein kinase isozymes in rabbit ovarian follicles and corpora lutea. *J. Biol. Chem.* **256**, 12185-12193.
Hunzicker-Dunn, M. and Mayo, K. (2006). Gonadotropin signaling in the ovary. In *Knobil and Neill's Physiology of Reproduction* (ed. J. D. Neill), 3rd edn, pp. 547-592. San Diego, CA: Elsevier/Academic Press.
Iancu, R. V., Ramamurthy, G., Warrier, S., Nikolaev, V. O., Lohse, M. J., Jones, S. W. and Harvey, R. D. (2008). Cytoplasmic cAMP concentrations in intact cardiac myocytes. *Am. J. Physiol. Cell Physiol.* **295**, C414-C422.
Jaffe, L. A. and Terasaki, M. (2004). Quantitative microinjection of oocytes, eggs, and embryos. *Methods Cell Biol.* **74**, 219-242.
Jaffe, L. A., Norris, R. P., Freudzon, M., Ratzan, W. J. and Mehlmann, L. M. (2009). Microinjection of follicle-enclosed mouse oocytes. *Methods Mol. Biol.* **518**, 157-173.
Johnson, M. T., Freeman, E. A., Gardner, D. K. and Hunt, P. A. (2007). Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. *Biol. Reprod.* **77**, 2-8.
Jones, K. T. (2008). Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. *Hum. Reprod. Update* **14**, 143-158.
Kalous, J., Solc, P., Baran, V., Kubelka, M., Schultz, R. M. and Motlik, J. (2006). PKB/AKT is involved in resumption of meiosis in mouse oocytes. *Biol. Cell* **98**, 111-123.
Kawamura, K., Ye, Y., Liang, C. G., Kawamura, N., Gelpke, M. S., Rauch, R., Tanaka, T. and Hsueh, A. J. W. (2009). Paracrine regulation of the resumption of oocyte meiosis by endothelin-1. *Dev. Biol.* **327**, 62-70.
Ledent, C., Demeestere, I., Blum, D., Petermans, J., Hamalainen, T., Smits, G. and Vassart, G. (2005). Premature ovarian aging in mice deficient for *Gpr3*. *Proc. Natl. Acad. Sci. USA* **102**, 8922-8926.
Locke, D., Stein, T., Davies, C., Morris, J., Harris, A. L., Evans, W. H., Monaghan, P. and Gusterson, B. (2004). Altered permeability and modulatory character of connexin channels during mammary gland development. *Exp. Cell Res.* **298**, 643-660.
Lugnier, C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. *Pharmacol. Ther.* **109**, 366-398.
Masciarelli, S., Horner, K., Liu, C., Park, S. H., Hinckley, M., Hockman, S., Nedachi, T., Jin, C., Conti, M. and Manganiello, V. (2004). Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. *J. Clin. Invest.* **114**, 196-205.
Mehlmann, L. M. (2005a). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* **130**, 791-799.
Mehlmann, L. M. (2005b). Oocyte-specific expression of *Gpr3* is required for the maintenance of meiotic arrest in mouse oocytes. *Dev. Biol.* **288**, 397-404.
Mehlmann, L. M., Jones, T. L. Z. and Jaffe, L. A. (2002). Meiotic arrest in the mouse follicle maintained by a G_s protein in the oocyte. *Science* **297**, 1343-1345.
Mehlmann, L. M., Saeki, Y., Tanaka, S., Brennan, T. J., Evsikov, A. V., Pendola, F. L., Knowles, B. B., Eppig, J. J. and Jaffe, L. A. (2004). The G_s-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* **306**, 1947-1950.
Mehlmann, L. M., Kalinowski, R. R., Ross, L. F., Hewlett, E. L. and Jaffe, L. A. (2006). Meiotic resumption in response to luteinizing hormone is independent of a G_i family G protein or calcium in the mouse oocyte. *Dev. Biol.* **299**, 345-355.
Newhall, K. J., Criniti, A. R., Cheah, C. S., Smith, K. C., Kafer, K. E., Burkart, A. D. and McKnight, G. S. (2006). Dynamic anchoring of PKA is essential during oocyte maturation. *Curr. Biol.* **16**, 321-327.
Nikolaev, V. O. and Lohse, M. J. (2006). Monitoring of cAMP synthesis and degradation in living cells. *Physiology* **21**, 86-92.
Nikolaev, V. O., Bunemann, M., Hein, L., Hannawacker, A. and Lohse, M. J. (2004). Novel single chain cAMP sensors for receptor-induced signal propagation. *J. Biol. Chem.* **279**, 37215-37218.
Nikolaev, V. O., Gambaryan, S. and Lohse, M. J. (2006). Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nat. Methods* **3**, 23-25.
Norris, R. P., Freudzon, L., Freudzon, M., Hand, A. R., Mehlmann, L. M. and Jaffe, L. A. (2007). A G_s-linked receptor maintains meiotic arrest in mouse oocytes, but luteinizing hormone does not cause meiotic resumption by terminating receptor-G_s signaling. *Dev. Biol.* **310**, 240-249.
Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., Lampe, P. D. and Jaffe, L. A. (2008). Luteinizing hormone causes MAPK-dependent phosphorylation and closure of Cx43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development* **135**, 3229-3238.
Panigone, S., Hsieh, M., Fu, M., Persani, L. and Conti, M. (2008). LH signaling in preovulatory follicles involves early activation of the EGFR pathway. *Mol. Endocrinol.* **22**, 924-936.
Patwardhan, V. V. and Lanthier, A. (1984). Cyclic GMP phosphodiesterase and guanylate cyclase activities in rabbit ovaries and the effect of in-vivo stimulation with LH. *J. Endocrinol.* **101**, 305-310.
Pincus, G. and Enzmann, E. V. (1935). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. *J. Exp. Med.* **62**, 665-675.
Richard, F. J., Tsafriri, A. and Conti, M. (2001). Role of phosphodiesterase type 3A in rat oocyte maturation. *Biol. Reprod.* **65**, 1444-1451.
Russwurm, M., Mullershausen, F., Friebe, A., Jäger, R., Russwurm, C. and Koesling, D. (2007). Design of fluorescence resonance energy transfer (FRET)-based cGMP indicators: a systematic approach. *Biochem. J.* **407**, 69-77.
Schultz, R. M., Montgomery, R. R. and Belanoff, J. R. (1983). Regulation of mouse oocyte meiotic maturation: Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev. Biol.* **97**, 264-273.
Sela-Abramovich, S., Chorev, E., Galiani, D. and N., Dekel. (2005). Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* **146**, 1236-1244.
Sela-Abramovich, S., Edry, I., Galiani, D., Nevo, N. and Dekel, N. (2006). Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology* **147**, 2280-2286.
Sela-Abramovich, S., Galiani, D., Nevo, N. and Dekel, N. (2008). Inhibition of rat oocyte maturation and ovulation by nitric oxide: mechanism of action. *Biol. Reprod.* **78**, 1111-1118.
Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. *Nature* **385**, 525-529.
Simon, A. M., Chen, H. and Jackson, C. L. (2006). Cx37 and Cx43 localize to zona pellucida in mouse ovarian follicle. *Cell Commun. Adhes.* **13**, 61-77.
Törnell, J., Billig, H. and Hillensjö, T. (1990a). Resumption of rat oocyte meiosis is paralleled by a decrease in guanosine 3′, 5′-cyclic monophosphate (cGMP) and is inhibited by microinjection of cGMP. *Acta Physiol. Scand.* **139**, 511-517.
Törnell, J., Carlsson, B. and Billig, H. (1990b). Atrial natriuretic peptide inhibits spontaneous rat oocyte maturation. *Endocrinology* **126**, 1504-1508.
Törnell, J., Billig, H. and Hillensjö, T. (1991). Regulation of oocyte maturation by changes in ovarian levels of cyclic nucleotides. *Hum. Reprod.* **6**, 411-422.
Viste, K., Kopperud, R. K., Christensen, A. E. and Doskeland, S. O. (2005). Substrate enhances the sensitivity of type I protein kinase A to cAMP. *J. Biol. Chem.* **280**, 13279-13284.
Vivarelli, E., Conti, M., DeFelici, M. and Siracusa, G. (1983). Meiotic resumption and intracellular cAMP levels in mouse oocytes treated with compounds which act on cAMP metabolism. *Cell Differ.* **12**, 271-276.
Webb, R. J., Marshall, F., Swann, K. and Carroll, J. (2002). Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase A in mammalian oocytes. *Dev. Biol.* **246**, 441-454.
Webb, R. J., Tinworth, L., Thomas, G. M. H., Zaccolo, M. and Carroll, J. (2008). Developmentally acquired PKA localization in mouse oocytes and embryos. *Dev. Biol.* **317**, 36-45.
Willoughby, D. and Cooper, D. M. F. (2008). Live-cell imaging of cAMP dynamics. *Nat. Methods* **5**, 29-36.
Wunder, F., Tersteegen, A., Rebmann, A., Erb, C., Fahrig, T. and Hendrix, M. (2005). Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. *Mol. Pharmacol.* **68**, 1775-1781.